The characterization of a novel C-type lectin-like receptor, CLEC9A

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Declaration

I, Cristal Huysamen, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgments indicate otherwise) and that neither the whole work or any part thereof has been, is being or is to be submitted for another degree in this or any other University.

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Cristal Huysamen

November 2008

This thesis is dedicated to my parents
Tobie and Corrie Huysamen

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Acknowledgements

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Abstract

I describe here the first characterization of CLEC9A, a group V C-type lectin-like receptor located in the "Dectin-1 cluster" of related receptors, which are encoded within the Natural Killer-gene complex. Expression of human CLEC9A is highly restricted in peripheral blood, being detected only on $BDCA3^+$ dendritic cells and on a small subset of $CD14^+CD16^-$ monocytes. CLEC9A is expressed at the cell surface as a glycosylated dimer and can mediate endocytosis, but not phagocytosis. CLEC9A possesses a cytoplasmic immunoreceptor-tyrosine based activation-like motif that can recruit Syk kinase and we demonstrate, using receptor chimeras, that this receptor can induce proinflammatory cytokine production. These data indicate that CLEC9A functions as an activation receptor.

Abbreviations

A

aa	Amino acid
Ab	Antibody
AGEs	Advanced glycation end-products
APC	Antigen presenting cell
AML	Acute myeloid leukemia
ASGPR	Asialoglycoprotein receptor

B

BGR	Beta-glucan receptor
β-gal	Beta-galactosidase
BSA	Bovine serum albumin

C

Ca^{2+}	Calcium
CLL-1	C-type lectin-like molecule-1
CHO	Chinese hamster ovary cell line
CNBr	Cyanogen bromide
CLB	Cell lysis buffer
CLECSF	C-type lectin superfamily
Clec2d	C-type lectin domain family 2 member d
CO_2	Carbon dioxide
CRD	Carbohydrate recognition domain
CTLD	C-type lectin domain
CTLRs	C-type lectin/lectin-like receptors

D

DAP10	DNAX-activating protein of 10kDa
DAP12	DNAX-activating protein of 12kDa
DCs	Dendritic cells
DCAL	DC-associated lectin
DCIR	Dendritic cell immunoreceptor
DC-SIGN	DC-specific ICAM-3 non-integrin
Dectin-1	DC-associated C-type lectin-1
dH_2O	Distilled water
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DMECs	Dermal microvascular endothelial cells
DNA	Deoxyribonucleic acid
DNGR-1	Dendritic cell NK lectin Group receptor-1
dNTPs	Deoxynucleotide triphosphates

E

ECD	Extracellular domain
ECL	Enhanced chemiluminescence
E coli	Escherichia coli

EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

F

FACS	Fluorescence activated cell sorting (flow cytometry)
Fc	Fragment crystallisable portion of IgG
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

G

G418	Geneticin/neomycin
gDNA	Genomic DNA
GE2	A monoclonal antibody against human BGR
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GlyCAM-1	Glycosylation-dependent cell adhesion molecule 1

H

HA	Haemagglutinin
HB3	A monoclonal antibody against human MICL
HI	Heat inactivated
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase

I

ICAM-2	Intracellular adhesion molecule 2
IFN	Interferon
Ig	Immunoglobulin
IgSF	Immunoglobin superfamily
IIDMM	Institute for Infectious Disease and Molecular Medicine
IL	Interleukin
ITAM	Immunoreceptor tyrosin-based activation motif
ITIM	Immunoreceptor tyrosin-based inhibitory motif

K

Kb(p)	Kilobase (pair)
kDa	Kilodolton
KIRs	Killer cell Ig-like receptors
KLRL1	Killer cell lectin-like receptor 1
KO	Knockout

L

LB	L broth
LDL	Low-density lipoprotein
LLR	Lectin-like receptors
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
LPS	Lipopolysaccharide

M

mAb	Monoclonal antibody
MHC	Major histocompatibility antigen
MICL	Myeloid inhibitory C-type lectin-like receptor
MGL	Macrophage galactose-type C-type lectin
MMR	Macrophage mannose receptor
MR	Mannose receptor
MW	Molecular weight
MΦ	Macrophage

N

NK	Natural killer
NKC	Natural killer gene complex
NKCL	Natural killer C-type lectin-like receptors
Nkrp1d	Natural killer cell lectin-like receptor subfamily B member 1 D

O

ORF	Open reading frame
Ox-LDL	Oxidised/acetylated LDL

P

pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
pH	Potential of Hydrogen
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorph-nuclear leukocytes
PNGase F	Peptide N-Glycosidase F
PRRs	Pattern recognition receptors
PTK	Protein tyrosine kinase
PV	Pervanadate

R

RNA	Ribonucleic acid
rpm	Revolutions per minute
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction

S

sLEX	Sialyl-Lewis X
SDS	Sodium dodecyl sulphate
SHIP	Src homology 2 domain-containing inositol 5-phosphatase
SHP	Src homology 2 domain-containing phosphatase
SR	Scavenger receptor
SSC	Side scatter
Syk	spleen tyrosine kinases

T

TLRs	Toll-like receptors
T_m	melting temperature
TNF-α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Rhodamine

U

UCT	University of Cape Town
UV	Ultra violet

V

V	Voltage

W

WT	Wild type

Symbols

˚C	Degrees Celsius
%	Percentage
α	Alpha
β	Beta
γ	Gamma
µ	micro
g	gram
g	gravity
M	Molar
mM	millimolar
mm	millimeter
ml	milliliter
ng	nanogram
U	units

List of Solutions

- **Cell lysis buffer (CLB) for extraction of genomic DNA:** 10mM Tris pH 7.8, 1000mM NaCl, 10mM EDTA pH 8, made up with dH_2O, 0.5% SDS was added thereafter
- **Coomassie staining solution:** 0.125% Coomassie Blue, 45.5% Methanol, 45.5% dH_2O, 9.2% acetic acid
- **Coupling buffer:** 0.1M $NaHCO_3$, 0.5M NaCl, dH_2O, pH8.3-8.5
- **Destainer for Coomassie:** 7.5% galacial acid, 5% methanol, 87.5% dH_2O
- **DNA loading dye (6x stock):** 0.05% Orange G, 30% (v/v) glycerol
- **FACS block:** PBS, 5% heat inactivated rabbit serum, 0.5% BSA, 5mM EDTA, 2mM NaN_3
- **FACS fixative:** 1% formaldehyde in PBS
- **FACS permeabilization buffer:** FACS block, 0.5% Saponin
- **Gey's solution [152]:** 20% A, 5% B, 5% C, 70% tissue culture grade dH_2O.
    - **Gey's solution A:** 3.5% NH_4Cl, 0.185% KCL, 0.15% $NA_2HPO_4.12H_2O$, 0.5% glucose
    - **Gey's solution B:** 0.42% $MgCl_2.6H_2O$, 0.34% $CaCl_2$, 0.14% $MgSO_4.7H_2O$
    - **Gey's solution C:** 2.25% $NaHCO_3$
- **HAT selection medium:** 20% fetal calf serum, 1x HAT medium in RPMI (containing 1000U/ug Penicillin/streptomycin and 20mM L-glutamine)
- **MOPS buffer:** 0.2M 3-[N-morpholino] propanesulfonic acid], 0.05M sodium acetate, 0.01M EDTA, pH5.5-7.0
- **Lidocaine/EDTA:** 4mg/ml Lidocaine, 10mM EDTA pH8 in PBS, filter sterilized
- **Lysis buffer for zymosan binding assay:** 3% Triton X100, dH_2O
- **NP-40 lysis buffer:** 1% Nonidet-P40, 0.15M NaCl, 10mM EDTA, 10mM NaN_3, 10mM Tris-HCl pH8
- **PBS:** 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH7.3
- **PBS-Tween wash buffer:** PBS, 0.1% Tween-20
- **Ponceau stain:** 0.2% ponceau powder, 5% acetic acid, made up in dH_2O

- **RNA loading buffer:** 48µl deionize formamide, 17.3µl 37% formaldehyde, 34.7µl loading dye
- **SDS-PAGE loading dye (2x stock):** 0.25M Tris pH6.8, 4% SDS, 20% glycerol, 0.001% bromophenol blue *reducing loading dye contains 10% 2-mercaptoethanol (2-ME), non-reducing contains no 2-ME
- **SDS-PAGE running buffer:** 25mM Tris, 90mM glycine, 0.1% SDS
- **Tris/EDTA (TE):** 10mM Tris pH8, 10mM EDTA pH8
- **Trypsin/EDTA:** 0.05% Trypsin, 5mM EDTA pH8 in PBS, filter sterilized
- **Western blot blocking buffer:** 2% (w/v) milkpowder, 0.5% BSA, 10mM NaN_3, PBS
- **Western blot transfer buffer:** 25mM Tris, 90mM glycine, 20% methanol
- **Lac Z phosphate buffer:** 0.1M Sodium phosphate dibasic, 0.1M Sodium phosphate monobasic, pH 8
- **Lac Z fix solution:** 0.25% gluteraldehyde, 0.5M EGTA pH7.3, 1M magnesium chloride, 0.1M sodium phosphate buffer
- **Lac Z wash buffer:** 1M magnesium chloride, 2% Nonidet-P40, 0.1M sodium phosphate buffer
- **Lac Z stain:** 2ml 25mg/ml X-gal stock dissolved in DMSO, 0.106g potassium ferrocyanide Sigma (P-9387), 0.082g potassium ferrocyanide Sigma (P-8131), 48ml Lac Z wash buffer

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Appendix for publications

1. **‘**CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3^{+} dendritic cells and a subset of monocytes’ **Cristal Huysamen**, Janet A Willment, Kevin M Dennehy, Gordon D Brown. (J Biol Chem. 2008 Apr 11).

2. ‘Characterisation of murine MICL (CLEC12A) and evidence for an endogenous ligand’ Elwira Pyż, **Cristal Huysamen**, Andrew S J Marshall, Siamon Gordon, Philip R Taylor, Gordon D Brown (Eur J Immunol. 2008 Mar 18).

3. ‘The Dectin-1 cluster of C-type lectin-like receptors’ **Cristal Huysamen**, Gordon D Brown (the manuscript has been submitted to FEMS Microbiology Letters)

Chapter One

Introduction

1.1 Cell surface receptors

Every host has specialised mechanisms to protect it from invading organisms and disease. Both the innate and adaptive immune responses depend upon the activities of leukocytes for an effective defense system. Leukocytes identify and eliminate pathogens, either by attacking larger pathogens through contact or engulfing and then killing them [1]. In order for these cells to execute their immune function they require a wide and flexible, yet specific and regulated, repertoire of cell surface receptors. Cell surface receptors are involved in leukocyte development, migration, differentiation, proliferation, maturation and survival; these receptors are also able to discriminate between host versus foreign molecules and eliminate aberrant host cells, such as cells that are transformed or virally infected; as well as mediating complex intracellular signaling cascades that coordinate various cellular responses. The majority of leukocyte cell surface receptors appear to be involved in mediating intracellular signaling responses [2].

Many cell surface receptors have the ability to recognize more than just one exogenous or endogenous ligand, associates with several intracellular molecules and engage in interactions with other transmembrane proteins. Leukocytes express a great number of different cell surface receptors [3], and the characterization of the function of each receptor is a clearly a complex task, but essential for understanding the basic biology of the immune system.

1.2 Receptor families and superfamilies

Cell surface receptors are classified into families or subfamilies based on their function, structural domains or conserved amino acid sequences. Family members that share structural homology may have related functions, however, it may also include members with non-conforming divergent functions. Receptors that are structurally homologous are usually derived from a common ancestor and are likely to have been generated by genetic changes such as exon shuffling, gene duplication and gene fusion [4].

The major structurally defined superfamily of cell surface receptors is the immunoglobulin superfamily (IgSF) commonly associated with roles in the immune system [5]. This family extends to the leukocyte cell surface where the immunoglobin (Ig) domain is found in approximately a third of known proteins and is involved in the recognition, binding or adhesion processes of cells [2]. There is a great number of other leukocyte cell surface protein families defined by their structural domains including integrins, complement control proteins, C-type lectins, G-protein coupled receptors and cytokine receptor domains. Some of these proteins contain more than one structurally defined domain and are capable of activating intracellular signaling cascades to regulate numerous cellular functions, for example phagocytosis, cytokine production and the synthesis of reactive oxygen species (ROS).

The pattern recognition receptors (PRRs) represent one of the functionally defined group of receptors, important to the innate immune system [6]. PRRs recognize microbial/pathogen structures, termed pathogen-associated molecular patterns (PAMPs),

which are characterized as being critical for pathogen survival, unrelated to host molecules and are often conserved amongst several mammalian species. The PRR concept explains how primitive organisms such as *Drosophila melanogaster*, which have no adaptive immune response, have the ability to combat a wide variety of pathogens with a relatively small repertoire of germ-line encoded immune receptors. PRRs are most notably represented by Toll-like receptors (TLRs), named after the fruit-fly receptor Toll, which was first discovered because of its important role in early fly development and later recognized as a major role player in innate immunity in adult flies [7]. Other PRRs include members of the C-type lectin superfamily, which recognize conserved pathogen-associated carbohydrates.

The mammalian genomes encode a variety of carbohydrate-binding molecules, which are collectively referred to as lectins [8]. This group of molecules is another superfamily of cellular proteins that can be defined by their structural domain and their intracellular signaling abilities. These proteins serve many different biological functions ranging from cell-cell adhesion, plasma glycoprotein turnover and innate pathogen recognition. Lectins play important roles in the immune system by recognizing carbohydrates at cell surfaces, on circulating proteins as well as those present on pathogens [9]. These circulating lectins include various soluble or transmembrane proteins. The cytoplasmic tails of most of these lectins are diverse and contain several conserved signaling motifs that are important for antigen uptake, intracellular signaling and the production of proinflammatory cytokines. The most diverse and well-studied family of lectins are the C-type lectins [10].

1.3 C-type lectins and lectin-like receptors

About 6% of leukocyte receptor proteins consist of C-type lectin receptors (CTLRs), which are involved in a variety of cellular responses [11]. C-type lectins contain a characteristic structural motif called the C-type lectin-like domain (CTLD), derived from six conserved cysteine residues that form three intra-chain disulfide bonds, which stabilizes the CTLD fold [12, 13].

This domain is a popular evolutionary framework for generating new functions and is found in various structural and functional contexts [14]. This family of proteins shows both evolutionary flexibility and conservation. For example, genomic studies have shown that there are virtually no structural similarities between the CTLD of *Caenorhabditis elegans* and the CTLD of *Drosophila melanogaster* [4], whereas relatively few modifications occurred within the vertebrate lineage during evolution from fish to mammals [15]. The only common feature between *Drosophila melanogaster* and *Caenorhabditis elegans* is the domain architecture consisting simply of an isolated CTLD [4, 16].

The CTLD was originally identified as a protein fold in the carbohydrate-recognition domain (CRD) of mannose binding lectin but since then it has been found in over 1,000 proteins [14]. In later studies the CTLD has been shown to specifically bind proteins [17], lipids [18], and inorganic compounds (only shown for invertebrates) [19-22]. In some cases the domain can bind both protein and sugar [23-25]. CTLD containing receptors include the ‘classical’ and ‘non-classical’ molecules, which have been classified into 17

groups or lectin subfamilies (Figure 1.1) based on the configuration of their CTLD(s) and the context amongst any other defined domains [14, 26]. The largest family of lectins are the 'classical' C-type lectins, and can bind carbohydrates in a Ca^{2+}-dependent manner [8]. The molecular mechanism of Ca^{2+}-dependent carbohydrate binding is conserved in all the family members studied up to date; the characteristic motifs 'EPN' and 'WND' form the core amino acids involved in this binding site. These motifs can be identified by sequence alignment and are indicative of the binding specificity (mannose vs. galactose) [14]. This observation provided a popular approach to predict whether the CTLD of an unknown function is likely to bind sugar (containing 'EPN' and 'WNP') or preferentially bind mannose- or galactose-type ligands ('EPN' vs. 'OPD' present) [14]. However, this approach is limited for the functional characterization of newly identified receptors, as its development was based on the comparison of a limited set of well-characterized domains and the number of uncharacterized sequences is quickly growing, including the evolutionary distance between the characterized and recently identified sequences.

The 'non-classical' C-type lectin receptors, also known as C-type lectin-like receptors, share structural homology with their classical counterparts, but lack the conserved residues in their CTLD involved in Ca^{2+}-binding [27]. These receptors have evolved to recognize non-carbohydrate ligands, however some may be able to recognize carbohydrates via alternative mechanisms [28-30]. All proteins containing a CTLD(s), including classical and non-classical will be collectively referred to here as 'C-type lectin receptors' (CTLRs).

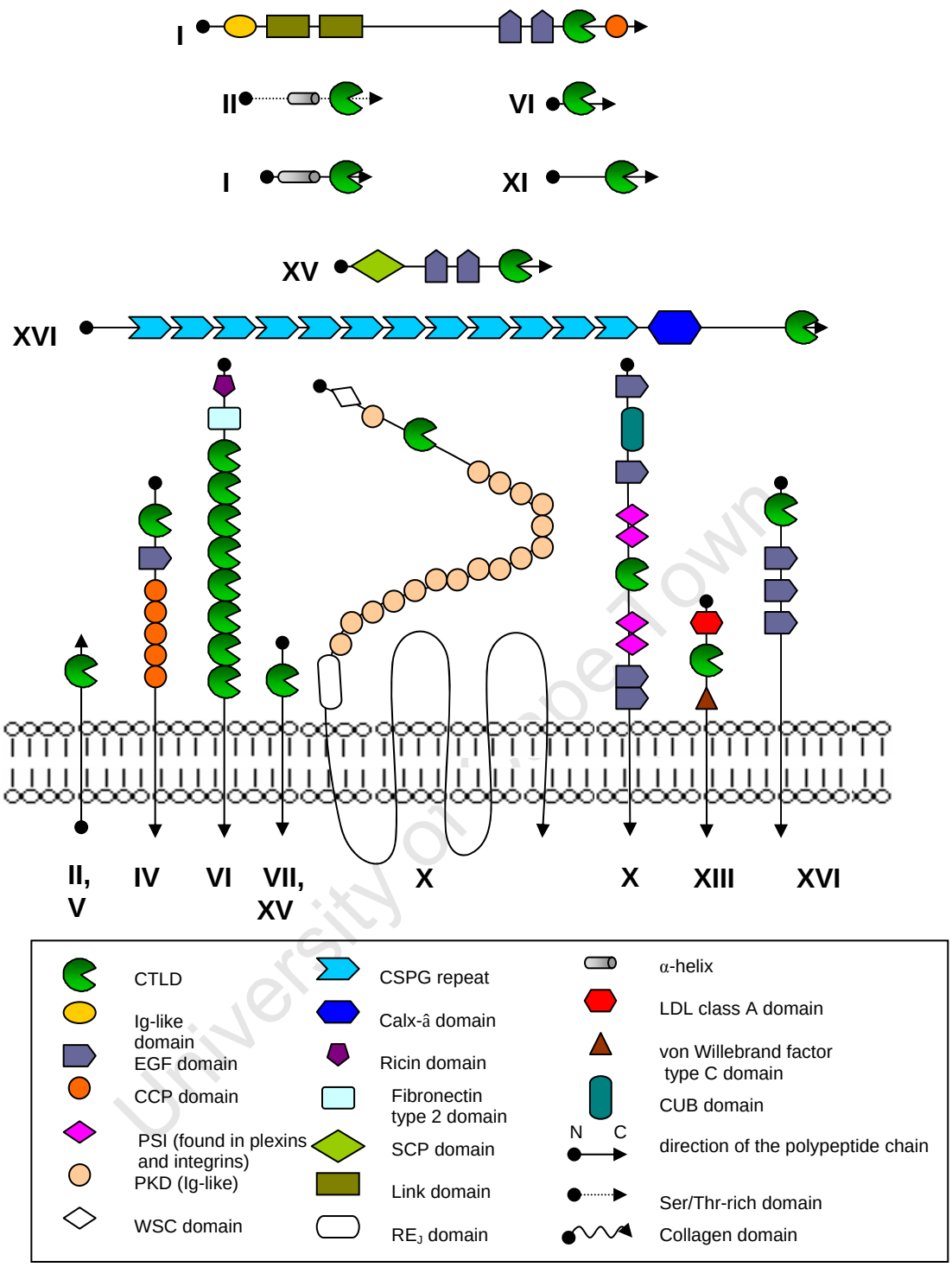


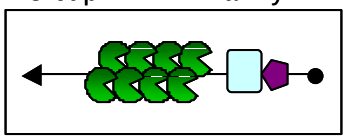
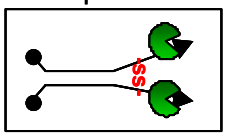
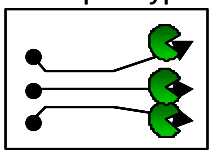
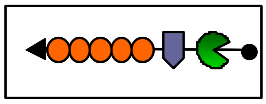
Fig.1.1 Domain architecture of vertebrate C-type lectin domains (CTLDs) from different groups. The C-type lectin family is separated into 17 groups based on their domain architecture. Group numbers are indicated next to the domain charts. I –lecticans, II – the ASGR group, III – collectins, IV – selectins, V – NK receptors, VI – the macrophage mannose receptor group, VII – REG proteins, VIII – the chondrolectin group, IX – the tetranectin group, X – polycystin 1, XI – attractin, XII – EMBP, XIII – DGCR2, XIV – the thrombomodulin group, XV – Bimlec, XVI –SEEC, XVII CBCP. Adapted from Zelensky and Gready (*FEBS Journal* 2005)

It is possible that the members of each of the 17 groups have evolved from one original CLTR, since the sequence of each CTLD is most similar to those within the same group [26]. The natural ligand(s) for the majority of individual members are still unknown and their biological function is yet to be defined. However, many of the characterized CTLRs are involved in various immune system functions, ranging from protective immunity through recognizing class I MHC molecules with self-peptides on healthy host cells to the killing of tumor or virally infected cells through their specific ligand recognition [31]. The functional versatility of this protein superfamily is owed to the diversity of ligands recognized by the CTLD.

1.4 The function of CTLRs in the immune system

Many lectin and lectin-like receptors are expressed on cells of the immune system which are important for the cellular functions of these cells [32]. As mentioned previously, this large family of receptors have been subdivided according to their structure and function (Figure 1.1) [14]. A summary of some of the major structural and functional relationships of the CTLRs and their role in the immune system is outlined in Table 1.1. A brief overview of the some of the important immune functions of selected CTLRs will be described below, as it is relevant to this thesis.

Table 1.1 Overview of the structural and functional relationship of selected subfamilies of C-type lectin-like receptors

Group and molecular structure	C-type lectin	Localization	Ligand specificity	Function	Cytoplasmic tail Motif (single letter amino acid code)
Group VI. MMR family 	MMR	DCs, LC, Mo, $M_{ø}$, LE	Mannose, fucose, sLe^x	Antigen uptake, Cell adhesion	FENTLY
	DEC-205 Endo 180	DMECs DCs, LC, tEC, Fibroblasts	Unknown Collagen	Antigen uptake EMC degradation	FSSVRY EDE FEGARY
Group V. NK receptors 	Dectin-1	DCs, $M_{ø}$, LC	β-glucan	Antgen uptake, Cell adhesion	YTQL DED
	CLEC-1	DCs	Unknown	Unknown	YSST DDD
	CLEC-2	DCs, platelets	Podoplanin	Activates platelets	YITL
	LOX-1	vEC, $M_{ø}$	Ox-LDL,	Phagocytosis	
Group II. Type II receptors 	DC-SIGN	DCs, HC, $dM_{ø}$, $aM_{ø}$	Mannan,Le^x, fucose	Antigen uptake, Cell adhesion	YKSL LL EEE
	DCIR	DCs, Mo, $M_{ø}$, PMN, B	Unknown	Unknown	ITYAEV
	Langerin	LC	Unknown	Birbeck granules formation	
	DCAL-1	DCs, germinal centre B	Unknown	T cell co-stimulation	
	BDCA-2	Plasmacytoid DCs	Unknown	Antigen uptake	EEE
Group IV. Selectins 	L-selectin	Leukocytes	$s6SLe^x$	Leukocyte tethering: Homing and inflammation	
	P-selectin	Platelets, endothelium	sLE^x,$s6Le^x$		YGVF
	E-selectin	Activated endothelium	sLE^x,$s6Le^x$		KKFV YQKP

Green sphere, C-type lectin domain; light blue rectangle, fibronectin type II repeat; purple pentogram, ricin domain; dark blue pentagon, epidermal growth factor (EGF)-like domain, orange oval, complement regulatory domain. Based on the nomenclature from Zelensky and Gready (*FEBS Journal* 2005). B, B cell; DCAL-1, DC-associated lectin-1; DMEC, dermal microvascular ECs; EMC, extracellular matrix; HC, Hofbaur cells; LC, Langerhans cells; LE, lymphatic endothelium; $M_{ø}$, d(decidual), a(alveolar) macrophages; Mo, monocytes; NK, natural killer; PMN, polymorphic nuclear cells; s6Lex, sialyl 6-sulpho Lewis x, tEC, thymic ECs; vEC, vascular ECs

1.4.1 The role of CTLRs in leukocyte trafficking

Several CTLRs have been characterized, which are expressed on leukocytes that play a role in cell trafficking. [33]. The selectins are of major importance in mediating cell adhesion and migration. This family comprises three members, E-, L- and P-selectins, and forms the prototype C-type lectin-like receptors [32]. The E- and P-selectin receptors are expressed on activated endothelium and play a major role in lymphocyte extravasations. The CTLD of both E- and P-selectin predominantly recognize the Sialyl-Lewis X (sLEX) carbohydrate, whereas L-selectin can bind CD34 and GlyCAM-1 (glycosylation-dependent cell adhesion molecule 1), expressed at low levels on endothelium.

The macrophage mannose receptor (MMR) can bind to macrophages and B cells through its cysteine-rich domain. This receptor has the ability to bind several classes of carbohydrates and provides a mechanism for regulating the trafficking and function of MMR-bearing cells [34]. MMR is also expressed on dermal microvascular endothelial cells (DMECs) and this has been associated with the diverse function of MMR, providing the first example of the dual function for a CTLR [32, 35, 36].

Evidence for a role of C-type lectins in trafficking dendritic cells (DCs) originates from the observation that intracellular adhesion molecule 2 (ICAM-2), constitutively expressed by endothelium supports tethering and rolling of cells expressing DC-specific ICAM-3 non-integrin (DC-SIGN) [37]. The DC-SIGN-ICAM-2 interactions regulate chemokine-

induced migration of DCs across both resting and activated endothelium *in vitro*, indicating that DC-SIGN is central to this trafficking capacity of DCs [37, 38]

1.4.2 Antigen presenting cell T-cell interactions

There is increasing evidence that CTLRs are important in the interactions that take place during the proliferation of leukocytes after antigen stimulation, which is associated with dense antigen-presenting cell (APC)-T-cell clusters. Its been previously shown that DC-SIGN is highly expressed on DCs, binds ICAM-3 and mediated transient adhesion of DCs with T cells [37]. This receptor supports the early antigen-nonspecific contact between DCs and T cells, enabling the engagement of T cell receptor by stabilizing the DC-T-cell contact zone [37].

Two other CTLRs, Dectin-1 [39] and DC-associated lectin-1 (DCAL-1) [40] are expressed on DCs and macrophages and have been proposed to bind T-cells. Fusion proteins of both proteins bind to the surface of T cells and promote their proliferation in the presence of anti-CD3 antibodies, suggesting that Dectin-1 and DCAL-1 on DCs bind to an undefined endogenous ligand on T cells leading to the delivering of T cell co-stimulatory signals.

1.4.3 Antigen uptake by CTLRs

The major function of C-type lectins in microbial recognition is the binding and internalization of microbes for direct elimination by macrophages [33]. Some well-known endocytic receptors include MMR, DEC-205 [41], BDCA-2 [42] and DC-SIGN

[43, 44] have also been shown to mediate antigen uptake. MMR delivers antigen to the early endosomes and recycles back to the surface, whereas DEC-205, BDCA-2 and DC-SIGN deliver antigens to the late endosomes or lysosomes, where they are degraded. These receptors contain various endocytic motifs in their cytoplasmic tail, including the tyrosine-based coated pit sequence uptake-motif present in MMR (YxxZ or FxxxxY, where X represents any amino acid and Z denotes any amino acid with a hydrophobic side chain) and the additional tri-acidic cluster (EEE or DDD) present in DEC-205, BDCA-2 and DC-SIGN, important for targeting to proteolytic vacuoles [41]. DC-SIGN has a di-leucine motif present in the cytoplasmic tail that is essential for internalization [43].

The ability of CTLRs to mediate endocytosis of ligands is not only important for pathogen clearance but at the same time also for the generation of antigenic fragments for presentation to T lymphocytes for triggering the adaptive immune system [33]. DEC-205, DC-SIGN and BDCA-2 mediate endocytosis and delivers antigen to late endosomal-lysosomal compartments of APCs and is an efficient means of generating MHC for presentation to T-cells [41-43, 45]. Antigen targeting to CTLRs expressed on APCs is emerging as a possible strategy for vaccination in humans and mice [46].

1.4.4 CTLRs and pathogen recognition

Several CTLRs are capable of recognizing PAMPs and play a role as phagocytic PRRs [32]. The role MMR as a PRR is well studied and it has been reported to bind a wide variety of pathogens including yeast [47], *Pneumocystis carinii* and *Mycobacterium*

tuberculosis [48]. The effect of these interactions to host defense is not fully understood although it has been reported that COS-1 cells transfected with MMR mediates phagocytosis of yeast and endocytosis of mannosylated BSA [47]. DC-SIGN binds numerous pathogens including viruses, fungi, bacteria, protozoa and products from metazoan parasites [49]. Dectin-1 and LOX-1 are both PRRs [29, 50], which recognize microbial components and can trigger numerous cellular responses including particle uptake [51, 52].

Although some CTLRs have been implicated in the recognition of pathogens, certain pathogens target CTLRs to escape immune activation. For example, DC-SIGN has a high affinity for the heavily glycosylated envelope glycoproteins of human immunodeficiency virus (HIV), however, DC-SIGN mediated uptake of HIV by DCs protect the virus from degradation and allow subsequent regurgitation and transmission to T cells [53, 54]. Similarly, the internalization of *Mycobacterium tuberculosis* by MMR aids in the survival of this pathogen [55]. These observations suggest that some CTLRs mediated pathogen binding and internalization may be misleading and may be indicative of CTLRs exploitation by the pathogen rather than pattern recognition leading to immune defense [46].

1.4.5 The role of CTLRs signaling

The function of Natural-killer C-type lectins (NKCLs) can be divided into activatory or inhibitory receptors, depending on the signaling motifs found in their cytoplasmic tails or their association with distinct signaling molecules. Inhibitory receptors possess generally

a conserved cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), whereas the activatory receptors possess a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM). Some activatory receptors contain a charged residue in their transmembrane domain, which can associate with an adaptor molecule (such as DAP10, DAP12, CD3ζ or FcRγ-chain) that possesses an ITAM in its cytoplasmic tail. However, it is possible that there is neither an obvious adaptor-associating residue nor a signaling motif, for example in the case of CLECSF8 [56] (C-type lectin superfamily member 8), which can still function endocytically.

The NKCL inhibitory and activatory receptors help regulate and maintain the functional balance of leukocytes [57]. Certain members of these inhibitory and activatory receptor superfamilies bear one or more cytoplasmic consensus motif (such as ITIMs and ITAMs) which have been shown to associate with specific signaling mechanisms, while other receptors signal via the adaptor molecules containing such motifs. The ITAM-mediated signaling mechanism involves a phophorylation cascade that differs between cell types through the use of different kinases within each family. The consensus sequence for ITAMs is $YxxLx_{5-12}Yx_{2-3}L/I$ [58]. Upon receptor stimulation the ITAM becomes tyrosine phosphorylated by members of the Src family tyrosine kinases, the phosphorylated ITAM then binds to Syk family kinases [59]. This tyrosine phosphorylation-based cascade continues through a variety of mediators. These ITAM-mediated activatory signals lead to a wide range of cellular outcomes, including phagocytosis, proliferation, adhesion and the production of various pro-inflammatory cytokines (Figure 1.2).

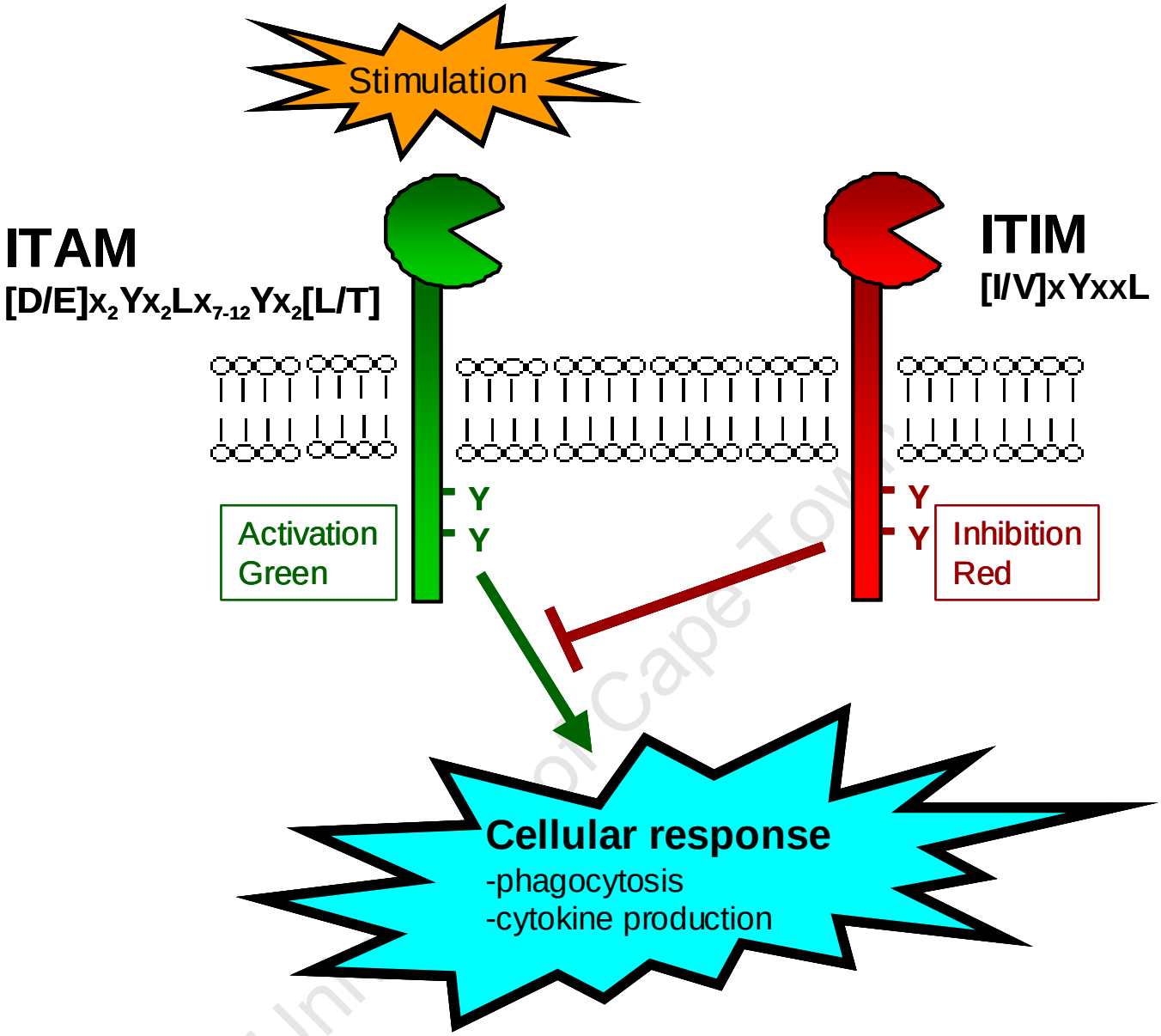


Fig.1.2 Activation and inhibitory receptors with tyrosine based motifs. Schematic representation of an activation receptor containing an ITAM, which becomes phosphoryated upon stimulation leading to various cellular responses including phagocytosis and cytokine production. The inhibitory receptor possess a ITIM which suppress activatory signals by recruiting phosphatases that dephosphorylate ITAMs.

ITIM-mediated inhibition involves dephosphorylation of the activatory cascades (Figure 1.2). The consensus sequence for ITIMs is I/V/L/SxYxxL/V [60]. The ITIMs are also phosphorylated by Src kinases [61] leading to the recruitment of phosphatase enzymes such as SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1) and potentially other phosphatases, such as SHP-2 and SH2-domain-containing inositol polyphosphate 5' phosphatases (SHIP) [60]. ITIM-mediated inhibitory effects include the inhibition of cell maturation, phagocytosis, cytokine production and cellular proliferation [60].

1.5 The Natural-Killer gene complex encoding lectin-like NK-receptors

The Natural-Killer gene complex (NKC) encompasses several families of genes encoding proteins that share a common structure [62]. The receptors encoded by these genes are primarily expressed on NK cells, therefore this genetic region is called the NK gene complex [63]. Most molecules encoded by the NKC are receptors that are highly relevant to NK-cell and T-cell function. The human NKC is located on the short arm of chromosome 12 and encompasses a region of about 2Mb [64]. In mice the corresponding region is located on chromosome 6 [65], which was originally found to contain the NKR-P1 and Ly49 genes [66]. The NKC contains numerous lectin-like receptor genes, of which some are expressed on NK and NK/T cells and function as NK receptors [67]. A novel family of genes, expressed on myeloid, DCs and or/ endothelial cells have recently become evident, serving as potential regulators of these cells [68].

NK cells are well recognized as playing an important role in the immune system in defending the host from invading pathogens and the rejection of tumor cells and

allogeneic cells [31]. The interactions of NK cell receptors with MHC class I and related molecules have provided insights into the mechanisms of how the immune system can detect and eliminate aberrant host cells, such as cells that are transformed or virally infected. Therefore this observation formed the basis for the 'missing-self' hypothesis, where NK cells survey tissues for the expression of MHC class I molecules [69]. In the absence of otherwise ubiquitously expressed MHC class I molecules, the result is the release of NK cells from the inhibitory influence of MHC class I molecules leading to elimination of the targeted cell (Figure 1.3) [70]. The NK cells respond to the 'missing self' as a signal of host stress and can be explained as the expression of NK-cell inhibitory receptors specific for MHC class I molecules [69]. These inhibitory receptors include human killer-cell immunoglobulin-like receptors (KIRs), the rodent Ly49 receptors and human and rodent CD94-NKG2 molecules [57, 63, 71, 72]. Another mechanism of detection is 'altered-self' recognition, in which inhibitory C-type lectin-like NK cell receptors (NKCLRs), e.g. Natural killer cell lectin-like receptor subfamily B member 1 D (Nkrp1d) that do not recognize MHC ligands, but other molecules including C-type lectin domain family 2 member d (Clec2d) [73], which is often downregulated on tumor cells [70].

The cytotoxic function of NK cells is regulated by these inhibitory and activation CTLRs. The functional balance of leukocytes other than NK cells are also regulated to a significant degree by lectin-like inhibitory and activatory receptors [57].

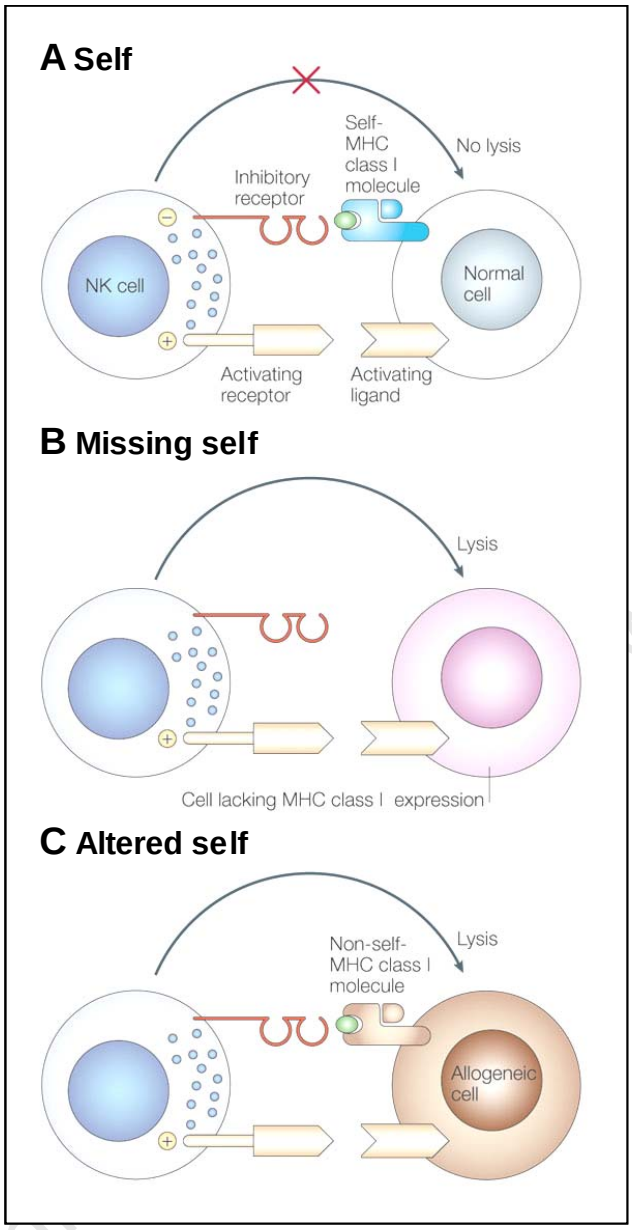


Fig 1.3 The interaction of NK receptors with MHC class I molecules. A) On interacting with a normal cell, a natural killer (NK) cell might receive activating signals; and because the target cell expresses the appropriate self-MHC class I alleles, the NK cell does not lyse the target cell. This is a consequence of inhibitory signals from the ligated MHC-binding receptor at the surface of the NK cell. **B)** If the target cell loses expression of MHC class I molecules, owing to viral infection/transformation, the MHC-binding inhibitory receptor at the surface of the NK cell is not engaged. Thereby, the NK cell does not receive inhibitory signals and therefore lyses the target cell. In this case, the target cell is perceived by the NK cell to be missing self. **C)** In the setting of an allogeneic transplant, host NK cells interact with donor target cells that express foreign MHC class I alleles. In most cases, the foreign, non-self-MHC class I molecules do not engage all of the inhibitory receptors at the surface of a host NK cell, leading to lysis of the allogeneic cells by host NK cells. Adopted from Kumar and McNerney (*Nature Reviews Immunology* 2005).

1.6 General features of the group V C-type lectin receptors

The NKC encode the group V CLTRs (Figure 1.1), which have been identified in higher vertebrates only [14]. They are usually encoded by six exons and share a common structure, forming dimeric type II transmembrane proteins with a single non-calcium binding CTLD in their ectodomain, a stalk region of variable length and a cytoplasmic tail, which may contain signaling motifs. Although the group V is defined as the "type II transmembrane NK cell receptors", many are not exclusively expressed on NK cells, but are also expressed on macrophages and DCs [39, 74]; vascular endothelial cells [75, 76]; and on platelets [77]. Thus, these CTLRs expressed on myeloid cells are in contrast to their NK cell counterparts, which primarily control cellular activation through the recognition of MHC class I and related molecules, therefore the myeloid-expressed receptors in group V appear to have more diverse functions [78]. These myeloid-expressed NKCLs play important roles in both innate and adaptive immunity through their ability to modulate myeloid cell functions [78].

Some of the group V receptors contain cysteine residues within their stalk region, which appear to be involved in homo- or hetero-dimerization. The human NKG2A-CD94 and NKG2C-CD94 form heterodimers capable of recognizing the non-classical MHC class I molecule HLA-E [79]. Multimerisation of these receptors increases ligand affinity and specificity [80].

CTLR gene expression includes: splicing, oligomerization, cell type restriction and regulation. Many CTLRs are alternatively spliced or even trans-spliced [81], though the

functional significance of this phenomenon is not well characterized. Many splice variants encode lectins that lack the stalk or transmembrane regions, leading to the retention of the receptor within the cytoplasm or the generation of soluble forms of the receptor, respectively [82, 83]. Alternative splicing may also mediate oligomerisation or aid in the flexibility of the CTLD [74, 84]. DCIR has stalked and stalkless isoforms that are oppositely regulated by anti- and pro-inflammatory stimuli [85].

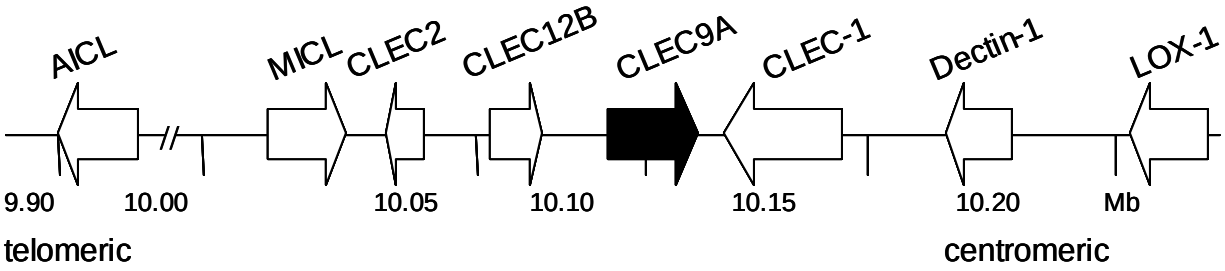
The regulation of CLTRs gene expression has also been well investigated. For example, various pro-inflammatory agents such as LPS [86] induce the expression of MINCLE, whereas LOX-1 [87] and the MMR [88] expression are upregulated by stimuli including anti- and pro-inflammatory cytokines. Soluble CTLRs such as collectins [89] and the cleavage of membrane bound CTLRs (e.g. MMR, CD23 and LOX-1) leads to the release of the receptor into the extracellular environment, which is another feature of expression that can be regulated [90]. These receptors perform various roles after being cleaved, such as opsonising pathogens for neutralization [91] or the clearance of host serum glycoproteins [34].

In the past decade the number of known activation and inhibitory NKCLs has increased considerably and many novel members are still being identified and characterized. The role of NK cell receptors appears to be primarily the identification of aberrant, transformed or virally infected cells, while the NKCLs appear to have far more divergent functions on myeloid cells. These receptors appear to have novel inhibitory and activatory mechanisms, but are mostly undefined. Little is known about their

physiological characterization *in vivo* and the identification of the ligand(s) of these receptors still remains a major challenge in this field. The future study of these receptors will give more insights into myeloid cell biology, and possibly provide alternative targets for the modulation of myeloid cell function for the treatment of diseases.

1.7 Dectin-1 cluster of C-type lectins

The 'Dectin-1 cluster' is a CTLR family within the NKC, which are predominantly, but not exclusively, expressed by myeloid cells and share common structures: type II transmembrane proteins whose extracellular domains have structural features of group V C-type lectins, a stalk region of variable length, and a cytoplasmic tail containing various signaling motifs. This cluster includes Dectin-1, LOX-1, CLEC-1, CLEC-2, MICL, CLEC12B and CLEC9A (Figure 1.4) and most of these receptors appear to have diverse ligands, cellular functions and are broadly expressed [78]. The 'Dectin-1 cluster' of receptors have been shown to recognize a broad range of structurally unrelated ligands, however, there are still a few receptors in this cluster that have not yet been functionally characterized and the natural ligand(s) are still unknown. The mechanism of the various intracellular signaling functions of these specific receptors is not clear in most cases, but the functions of the 'Dectin-1 cluster' indicates that these molecules may contribute to both innate and adaptive immune responses as well as homeostasis. Therefore we will focus on describing the receptors of this cluster in detail, as it will develop a better understanding of the key issues related to this thesis.



<u>Fig. 1.4</u> The genomic localization of the "Dectin-1cluster" of C-type lectin receptors. Schematic view of the "Dectin-1 cluster" on human chromosome 12p13, indicating the position of CLEC9A (black arrow) and the other members of this cluster.

1.7.1 Dectin-1 (CLEC7A)

Dectin-1 was originally reported as a cell surface Dendritic cell-specific molecule, from which its name 'dendritic-cell-associated-C-type lectin-1' was derived [39], but was subsequently found in a functional screen of a macrophage derived cDNA expression library to be a β-glucan receptor [29]. The expression of Dectin-1 is predominant in monocytes, macrophages, neutrophils and microglia, and to a lesser extent in a subset of T cells, and, in humans, B cells, mast cells and eosinophils [92-95]. The mouse Dectin-1 receptor is expressed at high levels in the lung and intestine representing the portals of entry for pathogens [92, 96]. Expression levels of Dectin-1 can be significantly influenced by a variety of cytokines and biological response modifiers such as β-glucan, GM-CSF and dexamethasone [93, 97, 98].

Dectin-1 has a similar structure to other CTLRs but in contrast to many of these receptors lacks cysteine residues in the stalk region, which are required for dimerization, indicating that the molecule is expressed, and functions as a monomer [39]. Dectin-1 is also N-glycosylated, like most CTLR, this post-translational modification contributes to the receptor's surface expression and function [99]. Both human and mouse Dectin-1 are alternatively spliced generating two major and a number of minor isoforms. The major isoforms possess altered functions and differ by the absence or presence of a stalk region [74, 100]. In a recent report, one of the minor human isoforms E (hDectin-1E) that lacks the stalk and transmembrane regions, was located in the cytoplasm and was shown to be capable of interacting with a cytoplasmic scaffold protein, Ran Binding Protein in the Microtubule-Organizing Center (RanBPM) [101].

Dectin-1 was the first example of an NKCL receptor whose primary ligand is a polysaccharide and not a protein [29]. Dectin-1 recognizes soluble and particulate β-glucans from fungi, plants and bacteria in a calcium-independent manner, and is the primary receptor for these carbohydrates on leukocytes [29, 102-104]. Although there are other receptors for β-glucans [105], Dectin-1 is the primary receptor for β-glucans on leucocytes [29, 106, 107]. Dectin-1 specifically recognizes 1,3-linked β-glucans and not monomers or polymers with other linkages [29, 108]. The crystal structure of the CTLD of Dectin-1 has been determined and mutational analyses have showed that two residues Trp^{221} and His^{223}, are essential for β-glucan binding, and these two residues flank a groove on the CTLD which could be a potential binding site [109, 110]. Structural analysis of this receptor revealed that Dectin-1 might dimerize through a novel interface, creating another binding site for β-glucan [110].

Dectin-1 has the ability to recognize other ligands on mycobacteria and on T-cells, which do not express β-glucans [111-113]. The recognition of T-cells by Dectin-1 is not inhibitable by β-glucans, suggesting that this receptor has two independent functional binding sites [74]. There Dectin-1 can stimulate activation and proliferation of T-cells, implying that it may function as a co-stimulatory molecule on APCs [39, 74, 114]. The expression of Dectin-1 by APCs in the T-cell area of lymphoid tissues supports the function of this receptor in these interactions [96]. The endogenous ligand of Dectin-1 has not been identified, although it may be a protein, which interacts through a distinct binding site on the CTLD of this receptor [39, 74]. Recently, Dectin-1 was shown to be

involved in the recognition and uptake of apoptotic cells and in the cross-presentation of cellular antigens [115].

Most of the cellular responses induced by Dectin-1 have been determined using zymosan, a particulate fungal-derived cell wall fraction from *Saccharomyces cerevisiae*. The function of Dectin-1 has been examined, more recently, by using highly purified β-glucans, such as curdlan and particulate glucan, there are also methods available to remove the contaminants from zymosan [103, 116, 117]. Utilizing these various β-glucans, shows that Dectin-1 induce ligand uptake by endocytosis and phagocytosis [51], the respiratory burst [106, 118], and the production of various cytokines and chemokines [103, 118-121]. Dectin-1 is sufficient for most of these responses, others, such as the production of inflammatory cytokines and the respiratory burst, however, are enhanced by the cooperative signaling from Myd88 coupled TLRs [118, 119, 122]. This was the first example of collaborative induction of cellular responses between a non-TLR and TLRs. Furthermore, Dectin-1 has the ability to induce cytokines, such as IL-23, which has been shown to directly couple the innate and adaptive immunity, independently from the TLRs [103].

The cytoplasmic tail of Dectin-1 contains an ITAM-like motif (YxxL), which resembles the tandem repeat sequence found in other activation molecules, such as DAP12, Fc receptors and lymphocyte antigen-receptor complexes [123]. The ITAM-like motif of Dectin-1 becomes tyrosine phosphorylated upon ligand binding by Src family kinases, which leads to the recruitment of Syk [51, 118, 120, 124]. Surprisingly, only the

membrane-proximal tyrosine within the ITAM-like sequence of Dectin-1 is required for signaling dexamethasone to require two phosphotyrosines for binding [51, 120]. Although the mechanism is still unclear, signaling is possibly initiated following the bridging of two Dectin-1 monomers [120, 124]. This was the first example of a single tyrosine based sequence being able to signal via Syk. This phenomenon has subsequently been shown to occur in other CTLRs, such as CLEC-2 [125]. The downstream signaling from Syk involves the novel adaptor CARD9, and activation of MAP kinases and NfκB [120, 126-128].

Syk is the central kinase involved in most of the Dectin-1 mediated cellular responses, but there are also Syk-independent pathways of the receptor signaling which may be cell-type specific. For example, Dectin-1 dependent phagocytosis in macrophages does not involve Syk, yet this kinase is partially required for particle uptake in DCs [51, 120]. The Syk-independent signaling is most likely involved in novel pathways, which are largely still uncharacterized [51].

The cell walls of fungi consist of up to 50% β-glucan, and the identification of Dectin-1 as a receptor for these carbohydrates suggested a role in anti-fungal immunity [129, 130]. Dectin-1 can recognize a number of fungal species including *Coccidiodes* [131], *Aspergillus* [132-134], *Candida* [119, 135], *Pneumocystis* [121] and *Saccharomyses* [119, 136]. Characterizing the interaction of these organisms *in vitro* with transfected and isolated primary cells, show that Dectin-1 induces a number of cellular responses,

including fungal internalisation and killing [121, 135, 137], cytokine and chemokine production [103, 119-121, 131, 132].

The recognition of fungi by Dectin-1 also triggers the production of non-protective cytokines, such as IL-10 and IL-23, the reasons for this are unclear [103, 120]. The induction of IL-23 contributes to the development of Th17 adaptive responses, which impair anti-fungal immunity by inhibiting protective Th1 responses and the promotion of inappropriate inflammation [138]. Dectin-1 induces the production of IL-10, this cytokine inhibits anti-fungal immunity, however the production of IL-10 through this pathway may be required to help reduce inflammatory pathology and to promote fungal persistence and long-term immunity [139-141].

The role of Dectin-1 *in vivo* has mixed results, which might be due to the use of knockout (KO) mice on different genetic backgrounds [142, 143]. On a 129Sv background, Dectin-1 KO mice, displayed increased susceptibility to systemic infection with *Candida albicans*, due to inflammatory defects and reduced fungal killing [107]. Conversely, Dectin-1 defiecient mice on a C57BL6 background were not susceptible to *Candida*, but there were some differences observed in fungal burdens during infection with *Pneumocystis carinii*, which resulted from defects in the respiratory burst [142]. The overall results support a role for Dectin-1 in anti-fungal immunity *in vivo*. The discovery of Dectin-1 and the study of its functions highlight a key role in innate anti-fungal immunity and is the first example of a signaling non-Toll-like PRR involved in the induction of protective immune responses.

The discovery of this receptor has provided some fundamental insights into the molecular mechanisms underlying the activity of β-glucans, and has extended the understanding of the functioning of the innate immune system. This receptor mediates various cellular functions, from fungal binding, uptake and killing to the induction of various cytokines and chemokines. The use of Syk to mediate the majority of these cellular responses has implications for the role of other CTLRs in immunity.

1.7.2 LOX-1 (CLEC8A, OLR1)

LOX-1 was initially discovered as an endothelial-specific scavenger receptor for oxidized low-density lipoproteins (Ox-LDL) and was the first member of the 'Dectin-1 cluster' to be identified [144]. The LOX-1 protein is well characterized and is the sole member of the class E scavenger receptor (SR) subgroup [145]. The extracellular domain of LOX-1 includes two N-glycosylation sites, three intra-molecular disulfide bonds, and two cleavage sites for membrane bound LOX-1, whereby soluble LOX-1 (sLOX-1) can also be released [146]. N-glycosylation of LOX-1, is a posttranslational modification which contributes to cell-surface expression and ligand recognition [147]. LOX-1 has the potential to form a homodimer through its disulfide bond, which promotes the multimerization of this receptor, enhancing ligand binding [148]. The C-type lectin-like fold of LOX-1 is highly conserved within the mammalian species [149].

The expression of LOX-1 on a cellular level has been observed in immature myeloid DCs, macrophages [76], monocytes, vascular smooth muscle cells [150], platelets and B cells [144, 146], and its expression can be upregulated by a variety of proinflammatory,

oxidative and mechanical stimuli during certain pathological conditions *in vivo*, including atherosclerosis, hypertension, hyperlipidemia and diabetes [151, 152]. Furthermore, the expression of LOX-1 can also be upregulated following inflammatory stimuli such as TNF-α, LPS, and fluid shear stress [75, 153]. Importantly, the binding of LOX-1 to Ox-LDL leads to the upregulation of receptor expression, which may aggravate the development of LOX-1 mediated diseases such as atherosclerosis [154].

LOX-1 recognizes multiple diverse ligands in addition to Ox-LDL, including gram-positive and gram-negative bacteria, such as *S.aureus* and *E.coli* [155], anionic polymers such as carrageenan [75], anionic phospholipids including phosphatidylserine [156], apoptotic and aged cells [52], activated platelets [157] and advanced glycation end-products (AGEs) [155]. The ligand recognition of LOX-1 is thought to involve electrostatic interactions with positively charged residues exposed on the surface of the CTLD of the receptor with negatively charged regions of the ligands [151, 158]. The diversity in ligands recognized by LOX-1 shows that this receptor has a versatile role in its physiological functions.

LOX-1 lacks the classical signaling motifs in its cytoplasmic tail, but can still mediate various cellular functions, including endocytosis, phagocytosis, cytokine production, apoptosis, the activation of NFκB and the production of ROS [151, 152, 158]. Additionally, LOX-1 can act as an cell-adhesion molecule involved in leukocyte recruitment during inflammation and through its ability to bind Hsp70 on human DCs,

LOX-1 is involved in Hsp70-mediated antigen cross presentation [159]. The signaling pathways leading to these cellular responses are not fully understood, although various downstream components have been implicated, including phosphoinositide 3-kinase, p38 mitogen activated protein kinase (MAPK) [158], protein kinase C (PKC) [30] and protein tyrosine kinase (PTK) [11] and transcription factor NF-κB [39]. In a recent study, LOX-1 mediated internalization of Ox-LDL was shown to occur through a clathrin-independent mechanism involving a novel cytoplasmic tri-peptide sequence motif of the receptor [160].

The major focus in LOX-1 was the interest in its involvement in vascular disease and the role of this receptor in the development of atherosclerosis. Various responses that are mediated by LOX-1 have been linked to the pathological, pro-atherogenic, changes in the vascular endothelium, and upregulation of LOX-1 in atherosclerotic lesions could result in a positive feedback system that promotes the development of atherosclerosis [151, 158]. In addition, increased release of soluble LOX-1 has been anticipated to be a marker for acute coronary syndrome. The LOX-1 KO mouse model supports a role for this receptor in the development of atherosclerosis [154, 161]. There is some evidence that LOX-1 may be involved in myocardial ischemia reperfusion injury, thrombosis, hypertension and may also be involved in generating inflammatory responses during microbial infection [158, 161].

LOX-1 has the ability to recognize a broad range of structurally diverse ligands, mediates various important immune functions despite lacking classical signaling motifs in its

cytoplasmic tail, and is involved in the development of various diseases. These special features of LOX-1 have extended the understanding of the diverse functional abilities of CTLRs and the importance of these receptors in homeostasis and immunity.

1.7.3 CLEC-1 (CLEC1A)

CLEC-1 and CLEC-2 were originally identified simultaneously as NKC-related lectins [162]. There is not much known about CLEC-1 in comparison with the other 'Dectin-1 cluster' receptors. CLEC-1 contains two extra cysteine residues in the CTLD, in addition to the six conserved cysteine residues. The stalk also contains a cysteine residue, although its not confirmed whether CLEC-1 is able to undergo homo- or heterodimerizations. Another interesting feature of CLEC-1 is the presence of a tyrosine residue in the predicted cytoplasmic domain. The intracellular tyrosine of CLEC-1 was found within the sequence YxxT and may represent a novel immunoreceptor tyrosine-based motif [46]. CLEC-1 mRNA expression is predominantly expressed in human placenta and lung and to a lesser extent in thymus and heart. High levels of CLEC-1 were detected by RT-PCR in DCs stimulated with TNF-α or by CD40-CD40 ligand interaction. CLEC-1 expression is absent in peripheral blood monocytes (PBMC), granulocytes, B-, T- or NK-cells and expressed to a lesser extent monocytes. CLEC-1 appears to be selectively expressed as at least two splice variants in activated DCs. In another independent study CLEC-1 mRNA was found to be significantly expressed in HUVEC (endothelial cell line derived from human umbilical vein endothelium) at a level comparable to primary DCs [68]. CLEC-1 cannot to be transported to the cell surface of transfected 293T cells. Therefore, it is possible that CLEC-1 may require an adaptor

molecule for its surface expression [162]. The ligand for CLEC-1 has not yet been identified and the physiological role of this receptor is yet to be determined.

1.7.4 CLEC-2 (CLEC1B)

This receptor was originally cloned from bone marrow and is preferentially transcribed primarily in the liver and PBMC, bone marrow cells, and myeloid cells [162]. The murine CLEC-2 shares 60% identity with the human homologue and is expressed on platelets and megakaryocytes [125, 162]. mCLEC-2 in platelets undergo differential glycosylation, consistent with the presence of three N-glycosylation sites [101]. Recently two novel splice variants of mCLEC-2 have been identified. The two variants are derived from the omission of exon 2 and 2/4, and each has a different expression profile and subcellular localization, in comparison with the full-length mCLEC-2 [163]. Additionally, the full-length mCLEC-2 has the ability to be cleaved into a soluble form, and both membrane-bound as well as soluble mCLEC-2 exist as homodimers [163].

The activation of CLEC-2 triggers platelet aggregation and activation, this activity is mediated by the ITAM-like motif of CLEC-2 [125]. This signaling motif resembles that found in Dectin-1, and can also recruit and signal via Syk [125]. In addition to Syk, the signaling pathway activated by CLEC-2 includes Tec family kineses, Src, PLCγ and Rac1 [124, 164].

An exogenous ligand of CLEC-1 has been identified as the snake venom toxin, rhodocytin [125]. Rhodocytin has the ability to form a tetramer which could activate and

induce CLEC-2 clustering to trigger downstream signaling and platelet activation [165-167]. CLEC-2 has also been shown to bind HIV-1 [101], and may capture and transfer infectious HIV-1 in cooperation with DC-SIGN [168].

Several studies have identified podoplanin, a mucin-type sialoglycoprotein, expressed on tumor cells as an endogenous ligand for CLEC-2 [77, 169]. A recent study showed that the monomeric extracellular domain of human CLEC-2 binds to podoplanin to a lesser extent than the multimeric one [169]. Dimerization or multimerization has been shown to be an efficient way to enhance the recognition of ligands for soluble and cell surface receptors [14]. The interaction of CLEC-2 with podoplanin is proposed to involve O-glycans, which may be involved in promoting tumor growth and/or metastasis, and could therefore be a possible target for therapeutic intervention [77, 169, 170].

CLEC-2 is the first CTLRs shown to activate platelets through a Syk-dependent manner, which is similar to the Dectin-1 signaling pathway for activation. This indicates that other CTLRs may also use similar signaling pathways for activation. Although endogenous and exogenous ligands for CLEC-2 have been identified, the physiological role of this receptor is yet to be defined.

1.7.5 MICL (CLEC12A)

MICL (CLEC12A) was identified as an inhibitory receptor, predominantly expressed on myeloid cells, granulocytes and monocytes as a heavily glycosylated monomer [171, 172]. MICL has been described by a number of groups under different names including

C-type lectin-like molecule-1 (CLL-1), Dendritic-cell-associated C-type lectin 2 (DCAL-2) and Killer cell lectin-like receptor 1 (KLRL1) [176, 177, 178]. The murine MICL receptor is structurally and functionally similar to the human orthologue, with some notable differences. mMICL is expressed as a dimer and is not glycosylated, but it is expressed primarily on myeloid cells like the human receptor [122]. Human MICL is variably spliced, generating at least three alternatively spliced isoforms identified in peripheral blood leukocytes (MICL-α, -β and –γ respectively) [171]. The expression of MICL is downregulated following inflammatory conditions, including responses triggered by microbial components [171, 172, 175]. MICL has also been identified as a marker for acute myeloid leukaemia in humans [176].

MICL can recruit inhibitory phosphatases (e.g. SHP-1 and SHP-2, but not SHIP) upon activation. These signaling events occur through the single ITIM sequence (VxYxxL) in the cytoplasmic tail of MICL and has the ability to inhibit cellular activation [171, 175, 177]. Although MICL is not expressed on NK cells, it has also been reported to inhibit NK cell cytotoxicity [177] and antibody mediated cross-linking of MICL on human monocytes-derived DCs has been shown to affect the function of DCs, by altering the cytokine production, cellular maturation and chemokine receptor expression, indicative of a role for this receptor in the control of immune responses [178].

There is some evidence for an endogenous ligand(s) for murine MICL and this was done by utilizing a cellular reporter system based on BWZ.36 cells, but the identity of this

molecule is still unknown [175]. The presence of an endogenous ligand(s) in multiple mouse tissues, suggests that MICL plays a role in homeostasis [175].

1.7.6 CLEC12B (Macrophage antigen-H)

CLEC12B (Macrophage antigen-H) was identified by its homology with NKG2D related molecules [179]. Unlike NKG2D however, the putative CLEC12B protein does not contain a charged amino acid in its transmembrane region but an ITIM sequence (VxYxxL) in the cytoplasmic tail [180], similar to that of MICL [171]. RT-PCR of CLEC12B in human cDNA libraries showed that this receptor is not expressed on primary T cells, NK cells or NK cell lines. The PCR results were obtained from an enhanced RT-PCR indicating that CLEC12B is expressed at very low levels. In mammary gland and ovary a truncated splice variant was found lacking exon 4, which is part of the CTLD, indicating that this alternative transcript could possibly yield a non-functional protein.

Human CLEC12B is expressed at the cell surface forming a homodimer in transfected cells. The protein expression of CLEC12B was found on *in vitro* differentiated macrophages and on PMA (Phorbol 12-myristate 13-acetate) stimulated monocytes (U937 cell line), but not on unstimulated cells. The ligand for CLEC12B has not yet been identified, however, it is able to function as an inhibitory receptor and is dependent on the ITIM sequence for this activity. CLEC12B signals via the recruitment of the phosphatases SHP-1 and SHP-2 to its ITIM upon receptor phosphorylation, using an

artificial model system, CLEC12B was able to inhibit certain cellular functions [179]. More functional analysis is required to understand the physiological role of CLEC12B.

1.8 CLEC9A

CLEC9A is a novel uncharacterized CTLR that is part of the 'Dectin-1 cluster' and is the focus of this thesis. Subsequently, two other independent publications on CLEC9A (called DNGR-1, Dendritic cell NK lectin Group receptor-1, by one group) appeared and the data from each of these publications will be mentioned and discussed throughout the thesis.

1.8.1 Aims of this thesis

The aim of this thesis was to characterize a novel group V CTLR, called CLEC9A, which is part of the 'Dectin-1 cluster', for both human and mouse, and to determine its functional role in immunity and/or homeostasis. The objectives were:

1. To make use of *in silico* analysis to determine the primary and secondary structure of CLEC9A and determine functional similarity CLEC9A might share with other known receptors,
2. To characterize the expression profile of the receptor,
3. To determine the intracellular signaling events and function of CLEC9A, and
4. To search for endogenous and exogenous ligand(s).

Chapter Two

Materials and Methods

2.1 Cells

2.1.1 Cell lines used

All cell lines were obtained from the IIDMM cell bank at UCT, and were cultured according to ATCC[a] recommendations. A summary of all the cell lines and their growth conditions used specifically for this study are listed in Table 2.1.

2.1.2 General cell culture

Each cell line was grown in the appropriate media and unless stated otherwise, media were supplemented with 10% heat-inactivated (HI) fetal calf serum (FCS) (from Sigma), 100units/ml penicillin, 0.1mg/ml streptomycin, and 2mM L-glutamine (referred to as RPMI^{10} or DMEM^{10}). Cells were cultured on tissue culture plastic flasks/plates in a 5% CO_2 humidified incubator, or in sealed, gassed flasks at 37˚C and routinely passaged after every 3 to 4 days in culture. Adherent cells were detached either with Trypsin/EDTA or Lidocaine/EDTA for 5 minutes at room temperature (RT).

[a] http://www.1gcpromochem.com/atcc/

Table 2.1 Summary of cell lines and their growth conditions

Cell line	Augmentation	Media used
A20: murine B lymphoma cells	Non-adherent	RPMI
EL-4: murine T lymphoma cells	Non-adherent	DMEM
BWZ.36NFAT-LacZ: reporter cells	Adherent	RPMI
NIH 3T3: murine embryonic fibroblasts	Adherent	DMEM
RAW 264.7: murine macrophage cell line derived from ascites of a murine intra-peritoneal tumor	Adherent	RPMI
293T: derivative of the human embryonic kidney 293 cell line	Adherent	DMEM
NS1: murine myeloma cell line	Non-adherent	RMPI
Y3: rat myeloma cell line	Non-adherent	RMPI
Phoenix eco (ÖNXeco): retroviral packaging cell line	Adherent	DMEM or RPMI
WT8: Syk-reconstituted B-cell line	Non-adherent	RPMI
C35: Syk-deficient B-cell line	Non-adherent	RPMI

2.1.3 Isolation and culture of human monocytes and *in vivo* derived macrophages/DCs

To obtain non-adherent mononuclear cells, monocytes, macrophages or DCs, 25ml of blood (obtained from Western Province Blood Transfusion Service, Cape Town, South Africa) was diluted 1:1 in room temperature PBS and 35ml of this was layered over 15ml solution of Ficoll-Hypaque (from Amersham) and spun at 900*g* at RT, for 20 minutes with no brake, 10ml serum was then removed, heat inactivated at 56˚C for 30 minutes, centrifuged at 1250*g* for 10 minutes, and 0.22μm filtered. The autologous serum was used at 1% in X-VIVO[10] medium (supplied supplemented with phenol-red and gentamycin) (from Invitrogen). The layer of mononuclear cells in the Ficoll-Hypaque was aspirated (including those adherent to the side of the tube), clumps were disrupted by vigorous pipetting and subsequently washed with ice cold PBS (made up to 50ml) pelleting at 500*g* for 7 minutes at 4˚C with low brake. The cells were washed 3-4 more times in PBS and once in RPMI, all with centrifugation at 250*g*, 4˚C for 5 minutes using full brake. The final pellet was resuspended in X-VIVO with serum and evenly spread on 15cm Petri dishes that had been pre-coated with 2% sterile gelatine (from Sigma). Following 90 minutes incubation at 37˚C, non-adherent mononuclear cells (mainly lymphocytes) were gently washed off with warm RPMI, 3 or 4 times. The monocytes remained adherent and were replaced in the incubator with fresh X-VIVO/serum overnight. Subsequently the monocytes were detached by gently washing with PBS and placed on ice. These monocytes were centrifuged at 350*g* for 10 minutes at 4˚C and resuspended in X-VIVO/serum, before counting viable cells using Trypan blue (from

Sigma), and plating for future experiments. For FACS analysis of macrophages and DCs, cells were plated in 48 well plates.

2.1.4 Isolation of peripheral blood leukocytes

For flow cytometric analysis of CLEC9A expression, 2ml peripheral blood was collected in 4mM EDTA to prevent coagulation, erythrocytes were lysed by adding 10ml ice cold fresh Gey's solution and 2ml FCS was then under laid [92]. The sample was centrifuged for 10 minutes at 300*g* with low brake, the supernatant discarded and the process repeated. The final pellet was resuspended in 1ml cold FACS wash and the cells were counted using Trypan blue.

2.2 General methods

All routine nucleic acid manipulation techniques were performed essentially as described by Sambrook, *et al.* [181].

2.2.1 Polymerase chain reaction (PCR)

All PCR reactions contained dATP, dTTP, dCTP and dGTP at 0.2mM each, appropriate primers at 0.2µM, 1.5mM MgCl, 1x manufacturer's PCR buffer (20mM Tris-HCl pH8.4, 50mM KCl: 10 x stock), 0.5 units Taq polymerase (from BD Bioscience) and ~100ng of template DNA per 20-50µl reaction and were incubated using a Gradient PTC-200 Peltier thermal cycler (from MH Research). Parameters for DNA amplification were dependent on primers and the template length, standard parameters involved a denaturing step at 94ºC for 2 minutes followed by 30-35 cycles of denaturation, annealing, and extension

(72ºC for 1.5 minutes per 1Kb of template). Annealing temperatures were set at 5ºC less than the melting temperature (T_m) of the annealing portion of the primer. After cycling, the reaction was incubated at 72ºC for a further 5 minutes and then maintained at 4ºC. Each reaction was analyzed by agarose gel electrophoresis or stored at -20ºC.

High fidelity PCR reaction was used to amplify cDNA for cloning using Advantage-HF2 polymerase (from Clontech) and the kit buffers according to the manufacturer's protocol. Cycling parameters were as above, but with extension at 68˚C.

2.2.2 Agarose gel electrophoresis

Agarose gels were prepared by dissolving electrophoresis grade agarose (from WhiteSci) under high temperature in 0.5 x TBE buffer to a final concentration of 0.8%. ethidium bromide (from Sigma) was added to the dissolved agarose to a final concentration of 0.2µg/ml. DNA loading dye was added to the samples before they were loaded onto the gel. DNA molecular weight marker was added for identification of fragments of specific size. Gels were run at a constant voltage of 90-100V, to give a good separation of fragments. The gels were analyzed with using a long wavelength (366nm) UV transilluminator (from UV Wirsam Scientific).

2.2.3 Purification of DNA from agarose gels

DNA fragments were excised from agarose gels using a long wavelength UV transilluminator, weighed and extracted from the agarose using SV gel and PCR purification kit (from Promega) according to the manufacturer's protocol.

2.2.4 Plasmid digestion

Digestion of plasmid vectors containing DNA inserts was preformed using appropriate restriction enzymes according to manufacturer's protocols and guidelines on the usage of each enzyme regarding temperatures and buffers. In general 25-50ng plasmid DNA was digested for 2 hours (or overnight) at 37ºC to ensure maximal digestion. The digestion reaction of some enzymes was terminated by heat inactivation of the enzyme at the appropriate temperature according to the manufacturer's protocol. In the situation when plasmids needed to be digested with two different restriction enzymes, manufacturer's protocols were consulted to determine whether both enzymes digestions could be performed in the same buffer. When this was not possible, sequential digestions were performed with the DNA being gel purified before the second digest was performed. The success of each digest was confirmed by gel electrophoresis, thereafter the desired insert or cut vector was cut out, and gel purified.

2.2.5 Plasmid dephosphorylation

Sometimes it was necessary to dephosphorylate cut vectors using 2U of shrimp alkaline phosphatase (from Fermentas) in the presence of manufacturer-supplied buffer to enhance the ligation. The reaction was incubated at 37°C for 60 minutes and then heat inactivated at 65°C before phenol/chloroform extraction.

2.2.6 Phenol/chloroform extraction of DNA

To remove protein contaminants, an equal volume of phenol was added to the nucleic acid sample and mixed by vortexing. Following centrifugation at 13,000rpm for 2

minutes, the aqueous phase was transferred to a sterile eppendorf tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phased and mixed by vortexing. Sample was centrifuged at 13,000rpm for 2 minutes and aqueous phase transferred to a clean 1.5ml eppendorf tube. The nucleic acid was precipitated using 0.1 volume of 3M sodium acetate, pH 5.2 and 2 volumes isopropanol and stored at -20˚C or pelleted by centrifugation at 13,000rpm for 15minutes at 4˚C and then washed in 70% ethanol to remove residual salts. After air-drying, the nucleic acid pellets were resuspended in TE buffer.

2.2.7 Ligation of plasmid vectors and insert DNA

For all ligation reactions, 25-50ng of plasmid was ligated with 50-250ng insert DNA in 20µl final volume containing 1U T4 DNA ligase (from Fermentas) and the manufacturers supplied buffer. Controls with no insert and/or no plasmid were included to observe false-positive background, to exclude self-ligation of vector or the presence of contaminating plasmids. Ligations were performed overnight at 16ºC. The ligation of PCR products was done by TOPO cloning (from Invitrogen) kit according to manufacturer's protocol.

2.2.8 Butanol precipitation

Butanol precipitation was performed after ligation for the removal of contaminants, which might influence the transformation competency. For each 20µl ligation mix 30µl dH_2O and 500µl butanol was added, vortexed for 10 seconds and centrifuged at 13,000rpm for 10 minutes at 4ºC. Supernatant was discarded and pellet containing plasmid DNA was air dried at 60ºC and resuspended in 2µl dH_2O.

2.2.9 Transformation and colony selection

Top10 competent *E. coli* (from Invitrogen) were stored at -80ºC until use. These cells were thawed on ice and kept on ice throughout the transformation process. A volume of 12µl competent cells was added to 2µl plasmid DNA and incubated on ice for 10 minutes. The cells were heat shocked at 42ºC for 30 seconds and placed back on ice before adding 250µl SOC. The reaction mix was incubated at 37ºC on a shaking platform at 300rpm for 45 minutes before being plated on pre warmed ampicillin-containing (100µg/ml) agar plates and incubated overnight at 37ºC.

All the vectors used contained an ampicillin resistant cassette to ensure that only those bacteria containing the successfully ligated plasmids could grow on the ampicillin containing agar plates and the bacteria that lack the ampicillin resistant cassettes do not. Incubation times were kept short and colonies were carefully selected to avoid satellite colonies. Colony PCR was used to identify colonies with correctly ligated inserts. Positive colonies were mini-prepped and tested by restriction enzyme digest and sent for sequencing.

2.2.10 Colony PCR

Colonies were screened by PCR prior to selection to confirm that the colony carried an insert in the desired orientation. Single colonies were sampled from ampicillin agar plates with a pipette tip, and transferred to a master plate and numbered for later identification of the ones yielding positive results. Thereafter the colonies were transferred directly to a tube containing 10µl PCR reaction mix using the same pipette tip. PCR was carried out

using the primers designed to amplify the insert of interest only if in the correct orientation. Colonies that tested positive for the desired insert were selected and inoculated for mini-prepping and sequencing.

2.2.11 Plasmid mini-prep

In order to generate enough plasmid DNA for analysis by restriction enzyme digest and sequencing, only the positive PCR colonies were selected for inoculation. Colonies were picked from the master plate with a pipette tip and added to 10ml LB broth containing 100µg/ml ampicilin and incubated at 37ºC shaking overnight. Aliquots of 0.6ml culture was resuspended with 30% glycerol and stored at -20ºC for future use. The remaining 9.4ml of the original culture was pelleted at 3,500rpm for 5 minutes and processed using a SV Mini-prep Kit (from Promega). Purified plasmid DNA was analyzed by sequencing to confirm the fidelity of the inserted sequence.

2.2.12 Isolation of genomic DNA

The genomic DNA was isolated from the newly generated cell lines to confirm that the cells were expressing the entire sequence introduced by retroviral transduction. Cells were detached with lidocaine/EDTA, pelleted at 850rpm for 5 minutes at 4ºC and lysed in cell lysis buffer (CLB), which had been supplemented with 50µg/ml Protease K (from Roche) immediately prior to use and lysates were incubated on a shaking platform at 55ºC overnight.

Genomic DNA was extracted by adding an equal volume of phenol to the lysates, followed by vigorous shaking and centrifuged for 2 minutes at 13,000rpm at 4ºC. Supernatants were removed and placed in new 1.5ml eppendorf tubes. An equal volume of chloroform:isoamyl alcohol was added and the tubes were inverted 4 times and centrifugation repeated. The aqueous phase was transferred to a new eppendorf tube, 1/10 volume 3M sodium acetate pH 5.2 was added followed by an equal volume of isopropanol. After a gentle shake the DNA was cooled at -20ºC to allow precipitation of DNA. Pelleted DNA was washed with 70% ethanol, air dried and resuspended in TE buffer prior to use. The sequence integrity was established by PCR using appropriate primers that flank the retroviral insert. Empty vector transduced cells served as a negative PCR control.

2.2.13 Isolation of RNA and reverse-transcription

Cells obtained after FACS-based sorting were snap frozen in liquid nitrogen and stored at -80°C, until ready for use. All pipettes and surfaces were cleaned with RNase-away (from Fluka). For every 100mg of tissue 1mL Trizol (from Invitrogen) was added and homogenized at RT. After 5 minutes at RT 0.2ml chloroform was added to the Trizol mix followed by vigorous shaking for 15 seconds and incubated for 3 minutes at RT. The samples were centrifuged at 12,000*g* for 15 minutes at 4°C. The aqueous phase containing the RNA was transferred to a new eppendorf tube and 0.5ml isopropyl alcohol was added and incubated at RT for 10 minutes to precipitate the RNA. The RNA pellet was washed with 70% ethanol, air dried and re-dissolved in RNase Free H_2O (from Promega). RNA concentrations were determined with the spectophotometer at OD

260nm. For long-term storage 0.25M sodium acetate and 2.5 times volume 100% ethanol was added to the RNA and stored at –80°C.

2.2.14 Formaldeyde gel electrophoresis gel

Formaldehyde gels were prepared by dissolving 1g electrophoresis grade agarose in 10ml 10x MOPS buffer. After the gel cooled down to RT, 5.4ml 37% formaldehyde was added and left to solidify in the fume hood. RNA loading dye was added to 5-15µg RNA and boiled for 2 minutes before it was loaded onto the gel. The gel was covered with 1x MOPS buffer containing ethidium bromide. Gels were run at a constant voltage of 90-100V, to give a good separation of fragments. The gels were analyzed using a long wavelength (366nm) UV transilluminator. The presence of 28S and 18S indicated the isolation yielded a good quality of RNA.

2.2.15 SDS-Polyacrylamide gel electrophoreses (PAGE)

Protein concentrations of cell extracts were measured at OD 280nm using the spectophotometer (from NanoDrop Technologies Inc.). Samples of approximately 25µg protein were mixed with sample buffer (reduced or non-reduced), boiled for 5 minutes at 95ºC, and 20µl per well were loaded into a 10% polyacrylamide stacking gel (pH 6.8) and resolved on a 10% acrylamide gel (pH 8.8) alongside molecular weight markers. Gels 1.5mm thick were cast and run in SDS running buffer using a Mini- PROTEAN III gel system (from BioRad) at a constant voltage of 100-200V as long as required for good separation. Gels were subsequently used in western blots, or analyzed by Coomasie staining for 30 minutes at 37ºC, followed by de-staining overnight.

2.2.16 Antibody purification and biotinylation

For the production of antibody stocks from hybridomas, cells were grown up in RPMI[20] and allowed to grow until the medium was exhausted. Supernatants were cleared of cellular debris by centrifugation at 1,000*g* for 10 minutes and were then 0.22µm filtered. Filtered supernatants were run over a Protein G Sepharose beads (from Amersham) column and fractions of purified antibody were eluted from the column using 2 volumes of 50mM glycine pH 2.5 into one volume of 1M Tris pH 8 to neutralize the elution buffer. The fractions containing the highest antibody concentrations were pooled, injected into a 10kDa cut off dialysis cassette (from Pierce) and dialysed overnight in PBS. Quantification of antibody concentration was repeated by using the spectophotometry and BCA protein assay (from Pierce). Purified antibodies were used at 10µg/ml and were tested by flow cytometry and Western blot.

A protocol supplied by S Cobbold (Oxford)[b] was used to biotinylate antibodies. Purified antibody was dialysed into bicarbonate buffer, and diluted to 1mg/ml. This was incubated with 37µl Sulfo-NHS-LC-Biotin (from Pierce) per ml in a glass bottle at 4ºC overnight. All conjugated antibodies were dialysed extensively into PBS and tested by flow cytometry before use.

2.2.17 Purification of polyclonal antibody

We generated a soluble Fc-mCLEC9A protein to immunize rats for the generation of polyclonal antibodies against the mCLEC9A receptor. The generation of this protein is described in section 2.5.3. The Fc-mCLEC9A soluble protein was dialysed in coupling

[b] http://users.path.ox.ac.uk/~scobbold/tig/fitc.html

buffer overnight at 4°C. For coupling, 1g cyanogen bromide resin (CNBr) (from Sigma) per 1mg Fc-mCLEC9A, was used. The resin was washed, using a Büchner, for 30 minutes with 200ml 1mM HCL, 250ml dH_2O and 5ml coupling buffer and transferred to the dialysed Fc-mCLEC9A protein to be rotated overnight at 4°C. All the unreacted ligand was washed away with coupling buffer and the resin was blocked in 0.2M glycin pH 8 for 2 hours at RT. The resin was washed extensively with coupling buffer, then 0.1M acetate buffer pH4 and lastly with 0.5M sodium chloride (NaCl) to remove the blocking solution.

The serum collected from immunized rats was rotated with 50µg/ml hIgG (from Sigma) overnight at 4ºC to compete for Fc reactivity from anti-mCLEC9A polyclonal anitbody. Thereafter the sample was spun at 3,000rpm for 15 minutes at 4ºC and filtered through a serum filter (from PALL, Life sciences). Filtered serum was run over the CNBr/Fc-mCLEC9A sepharose column and fractions of purified antibody were eluted from the column using 2 volumes of 50mM glycine pH 2.5 into one volume of 1M Tris pH 8 to neutralize the elution buffer. The elution fractions were measured at analyzed OD 280nm. The fractions containing the highest antibody concentrations were pooled, injected into a 10kDa cut off dialysis cassette and dialysed overnight in PBS. Quantification of antibody concentration was repeated using the spectophotometry and BCA protein assay (Pierce). Purified antibody was used at 10µg/ml and was tested by flow cytometry and β-galactosidase assay.

2.3 Transfection/transduction of cell lines and flow cytometry

2.3.1 Transient transfection using Fugene 6

The Fc-fusion plasmids (constructs described in section 2.6.2) were transfected into HEK293T cells using Fugene 6 (from Roche) according to the manufacturer's protocol. Cells were plated out at 1 x 10^6 in 3mL $DMEM^{10}$ the day prior to the transfection in a 6 well plate. Supernatants were discarded and 2ml fresh $DMEM^{10}$ was added. Cells were freshly cultured to 60% confluence and the transfections were performed in $DMEM^{10}$. Trail transfections were usually done in 6 well plates but for the generation of large quantities of recombinant protein, T175 flasks were used and kept under 0.4mg/ml Zeocin (from Invitrogen) selection.

2.3.2 Retrovirus production

Inserts were excised from cloning vectors by restriction enzyme digestion and ligated into a retroviral vector, pFBneo (from Stratagene). Following ligation, colony selection and sequencing, vectors were transfected into a packaging cell line, Phoenix eco (ΦNXeco).[c] On Day 1 recently passaged ΦNXeco packaging cells were plated at 2x10^6 per well in 6 well plates overnight in 3ml $DMEM^{10}$ medium. The following day (Day 2) the medium was gently replaced to avoid detaching cells, to a final volume of 2ml per well. Packaging cells were then transfected according to the manufacturer's protocol with Fugene 6 using 1µg DNA and 6µl Fugene per well (initially mixed with 94µl DMEM without serum) and incubated overnight at 37ºC. On Day 3, plates containing the

[c] http://www.stanford.edu/group/nolan/protocals/pro_optimiz.html

transfected cells were transferred to a 32ºC incubator in a CO_2-pulsed airtight container. On Day 4, supernatants were harvested and 5µg/ml polybrene (from Sigma) was added to reduce electrostatic repulsive forces between virus and target cell. Supernatants were then filtered through a 0.45µm filter and aliquots of 2ml were used immediately or stored in cryovials at -80ºC prior to use.

2.3.3 Retroviral transduction and selection of cell lines

On Day 1, target cells were plated at $2x10^6$ for NIH3T3 or $2x10^5$ for RAW264.7 cells (pre-treated with 0.2µg/ml tunicamycin (from Sigma) to enhance transduction efficiency) per well in 6 well plates overnight. The following morning, Day 2, cells were washed using fresh medium and replaced with 1.5ml of the filtered viral supernatants. Plates were then spun at 2,500rpm at 25ºC for 90 minutes to increase transduction efficiency [74]. Plates were then returned to the 37ºC overnight. On Day 3 the viral supernatants were replaced with fresh $DMEM^{10}$ and on Day 4 the medium was replaced with selection medium: $DMEM^{10}$ containing 600µg/ml G418. Cells were kept under selection until transduced colonies became clearly visible before transferring the cells to flasks.

2.3.4 Flow cytometry

In the majority of assays, cells were stained prior to fixation (referred to as 'live' and unpermeabilised) to preserve sensitive epitopes and to analyse only cell surface markers. Staining was performed at 4ºC to limit membrane mobility and cells were blocked with FACS block buffer to reduce non-specific staining. In experiments using RAW264.7 cells, the anti-FcγRII/III antibody (2.4G2) was added to FACS block to reduce non-

specific background staining. Cells were either stained when still adherent (in 24 or 48 well plates) or were first lifted with lidocaine/EDTA and then stained in V-bottomed 96-well plates. All primary antibodies were used at 10µg/ml and cells were washed three times with FACS wash between staining steps. Secondary antibodies were used at between 1:100 and 1:200 dilutions. All monoclonal antibodies used were compared with staining by isotype-matched control antibodies. Incubation times were typically 60 minutes for primary antibody and 45 minutes for secondary antibody staining. Stained cells were usually fixed in 1% formaldehyde prior to flow cytometry. Data were analyzed using Cellquest (from BD Bioscience).

For fix-permeabilized flow cytometry staining, the cells were treated with 1% formaldehyde at 4°C for 30 minutes, before blocking with FACS block containing 0.05% saponin (from Sigma) for 30 minutes prior to the addition of antibodies. All primary antibodies were used at 10µg/ml and cells were washed three times with FACS wash between staining steps. Secondary antibodies were used at between 1:100 and 1:200 dilutions. All monoclonal antibodies used were compared with staining by isotype-matched control antibodies.

2.4 Techniques specific to this study

2.4.1 Computer tools

Sequence analysis was performed using various on-line tools[d]. Sequences were aligned with Clustal X [182], dendrogram analysis and percentage identities were calculated by DNAMAN Version 4.0 (from Lynnon BioSoft). All graphs were generated in Microsoft Exel or GraphPad Prism, and all FACS analyses were performed using Cell Quest. Figures were compiled in Microsoft PowerPoint, and text in Microsoft Word. Images were processed using Adobe Photoshop version 6.0 and Multi-Analyst.

2.4.2 Generation of cell lines and transduced cell lines

The complete CLEC9A open reading frame (ORF) was isolated from human PBMC cDNA by PCR and cloned into the pFBneo (from Stratagene) retroviral vector containing an HA-tag [171] using the following primers: **5'**AAAGAATTCCCACCATGCACGAGGA AGAAATATAC**3'** and **5'**AAACTCGAGGACAGAGGATCTCAACGC**3'**.

C-terminally HA-tagged murine CLEC9A (mCLEC9A) was similarly cloned from mouse splenic cDNA using the following primers: **5'**AAAGTCGACCACCATGCATGC GGAAGAAATA**3'** and **5'**GTACTCGACGATGCAGGATCCAAATGC**3'**.

The CLEC9A/Dectin-1 chimera in pFBneo was generated using overlap extension PCR with primers: **5'**CTGCTAAACTTTACAGAAAACCAC-AAGCCCACA**3'** and **5'**TCTGGGCTTGTGGTTTTCTGTAAAGTTTAGCAG**3'** such that the sequence translated as $^{CLEC9A-79}$LLNFTE$^{CLEC9A-84}$/ $^{Dectin-91}$NHKPT$^{Dectin-95}$.

[d] http:ca.expaxy.org
http://www.ncbi.nlm.nih.gov/BLAST/
http://ncbi.nlm.nih.gov/entrez

The Fc-hCLEC9A expression construct was generated by PCR using the following primers: **5'**TTTGGTACCAGCAGCAAGAAAAACTC**3'** and **5'**GCGGAATTCGAC AGAGGATCTCAACGC**3'**. Fc-mCLEC9A expression construct was generated using the following primers: **5'**AAATGGTACCAGCAGGAAAGACTCATC**3'** and **5'**AAAGAATTCGATGCAGGATCCAAATGC**3'**, both constructs were cloned into the pSecTag2 vector (from Invitrogen) upstream from the human IgG1 Fc region, generated as described [183].

The fidelity of all constructs was verified by sequencing. Human and murine CLEC9A and isoforms have been deposited at GenBank under the following accession numbers: hCLEC9A, EU339276; mCLEC9A, EU339277; mCLEC9Aβ, EU339278; mCLEC9Aγ, EU339279; mCLEC9Aδ, EU339280; mCLEC9Aε, EU339281.

To generate stable cell lines, constructs were packaged into virons, using HEK293T based Phoenix ecotropic cells, and the various cell lines were transduced as previously described in section 2.3.2. All cell lines were used as non-clonal populations to reduce founder effects and were generated and tested at least twice to confirm phenotype. Where required, cell lines were selected and maintained in 0.6mg/ml G418 or 4μg/ml puromycin (from Invitrogen).

Human CLEC9A expression was analyzed by PCR of human tissue cDNA panels (from Clontech) using the following primers encoding the entire ORF, **5'**ATGCACGAGGAA GAAATATACACC**3'** and **5'**GACAGAGGATCTCAACGCATA**3'**. mCLEC9A expression

was analyzed by PCR of mouse tissue cDNA panels (Clontech) using the same murine primers described above. Expression of G3PDH was used as a positive control using the following primers, **5'**TGAAGGTCGGAGTCAACGGATTTG**3'** and **5'**CATGTGGGCCATG AGGTCCACCAC**3'**.

2.4.3 The generation and testing of monoclonal antibodies (mAb)

An eukaryotically-derived Fc-CLEC9A recombinant fusion protein was generated as an antigen for mAb generation for both human and murine CLEC9A as described above. This PCR product was obtained using the Fc specific primers and cloned into the KpnI/EcoRI sites of pSecTag2(C) vector (from Invitrogen) upstream of an 'Fc^{mut}' construct designed by Dr. J Willment (UCT). This human Fc region does not fix complement or bind Fc receptors [184]. This was transiently transfected into 293T cells using Fugene 6 and viral packaging cells, and the supernatant harvested. The medium on the cells was replaced with medium containing zeocin (from Sigma) and a stable clone producing high levels of Fc-CLEC9A was isolated by clonal dilution and tested by anti-Fc dot blotting. The soluble protein was isolated from the supernatants on a protein A column, extensively dialysed into tissue-cultured grade PBS and quantified by spectophotometry. The fusion proteins were used for the immunization of three BalbC mice (Fc-hCLEC9A) and three Wistar rats (Fc-mCLEC9A) between 4-6 weeks old.

Immunization was performed by H Arendse (UCT) according to standard protocols [185]. A pre-bleed sample was taken to estimate background titer. Approximately 10μg Fc-hCLEC9A per mouse and 20μg Fc-mCLEC9A per rat was homogenized with 75%

sterile Fc-protein in sterile PBS and 25% Titremax Gold (from Sigma), and injected intra-peritoneally. Two booster injections at 2 and 4 weeks later were administered and four days prior to splenocyte harvesting/fusion, 30μg Fc-CLEC9A was injected intravenously in PBS.

A total of $5x10^7$ NS1 myeloma cells were resuspended in 10ml RPMI (no serum) and kept at RT. The spleen was harvested and kept on ice in RMPI (no serum). Splenocytes were isolated by homogenizing the spleen through grinding it between two frosted glass slides with a circular motion to release splenocytes. The spleen cell suspention was filtered through a BD falcon cell strainer (from BD Bioscience) and collected into a 50ml falcon tube filled with RPMI (no serum). The splenocytes were washed twice with centrifugation at 1,200rpm at 25°C for 5 minutes. Approximately 1 $x10^8$ splenocytes were counted and resuspended in 10ml RPMI (no serum). The $5x10^7$ myeloma cells were mixed with the $1x10^8$ splenocytes to a total volume of 50ml RPMI (no serum) and centrifuged at 1,200rpm at 1,200rpm at 25°C for 5 minutes. The supernatant was discared and the tube containing the pellet was placed in a beaker water bath at 37°C. During the first 30 seconds 1.5ml of 50% polyethylene glycol (PEG) 1500 (from Sigma) was added drop wise, while agitating cell mixture by flicking the tube with finger throughout the whole procedure. The pellet was resuspended by gently pipetting for 30 seconds and after waiting for another 30 seconds 1 ml RPMI (no serum) was slowly added within 1 minute, this was the most critical step. Another 4ml RMPI (no serum) was slowly added within 2 minutes, followed by slowly adding 5ml RPMI (no serum) over the next two minutes. The cell mixture was centrifuged at 1,200rpm at 25°C for 5 minutes. The supernatant was

discarded and the pellet resuspended in 100ml HAT selection medium. A 100µl of fused cells were dispensed per well in 10 x 96well/plates and feed with another 100µl/well HAT medium immediately or the next day for a final volume of 200µl/well. On day 3, 5 and 7 after the fusion a 100µl of medium was removed from each well and replaced with 100µl HAT selection medium. Some wells were be ready by day 10, the majority cells were screened by day 14 after the fusion.

Following the generation of clonally diluted NS1 hybridomas, the supernatants of each hybridoma were screened by enzyme linked immunofluorescent assay (ELISA). A control Fc-hDectin-1 protein was provided by Dr. J Willment (UCT) (this soluble protein is designed similarly to Fc-hCLEC9A) and Fc-hCLEC9A soluble protein was coated on the ELISA plates at 1µg/ml overnight at 4˚C. The plates were then washed with PBS-Tween 0.5% and blocked with 0.5% BSA at 37˚C for 2 hours. After washing, 50µl hybridoma supernatant was added and incubated at 37˚C for 2 hours before washing and then detection with an alkaline phosphatase conjugated anti-murine secondary antibody (from Jackson).

2.4.4 Western blotting and deglycosylation

To prepare cellular extracts, cells were washed with PBS and then lysed in cell lysis buffer (CLB), supplemented with protease inhibitors (from Roche), for 30 minutes at 4°C. Cellular debris/nuclei were removed by centrifugation, and supernatants were collected and stored at -20°C. Protein concentrations of cell extracts were determined by BCA assay (from Pierce) and equal quantities of protein were run on SDS-PAGE gels,

according to standard protocols. After transfer to HybondC+ nitrocellulose membranes (from Amersham), CLEC9A was detected by immunostaining with 9A11 or anti-HA (clone 16B12, from Covance) followed by goat anti-mouse HRP (from Jackson). Blots were developed with the ECL-plus kit (from Amersham).

NIH3T3 transfected fibroblasts were lysed in CLB, supplemented with protease inhibitors, for 30 minutes at 4°C. Cellular debris/nuclei were removed by centrifugation, and supernatants were collected and deglycosylated with PNase F and/or O-glycosidase (from Roche), as described by the manufacturer.

2.4.5 Cell sorting

The following antibodies were used in these experiments: 9A11-biotin, D1.3-biotin (a gift from Dr L. Martinez-Pomares, Oxford University), CD1a, CD2, CD3- fluorescein isothiocyanate (FITC) (Serotec), CD4-phycoerythrin (PE) (Serotec), CD8-PE (Serotec), CD11b-FITC (Serotec), CD11c-FITC (Serotec), CD14-FITC (BD-Pharmingen), CD16-PE (BD Pharmingen), CD19-PE (BD-Pharmingen), CD56-PE (BD-Pharmingen), CD83-PE (Serotec), CD86-PE (Serotec), BDCA-1-FITC (BD Pharmingen), BDCA-2-PE (BD Pharmingen), DC-SIGN-FITC (BD Pharmingen), HLA-DR-FITC (BD Pharmingen), BDCA3-PE (Miltenyi) and irrelevant biotin-, PE- or FITC-labeled mouse IgG1 (D1.3) IgG2a (BD Pharmingen) and IgG2b (BD Pharmingen) control antibodies. Biotinylated antibodies were detected using streptavidin-allophycocyanin (APC; BD Pharmingen).

To isolate $9A11^+$ cells for analysis, 30 million PBMCs, isolated as described above, were blocked with 5% mouse serum or 50μg/ml human IgG for 15 minutes at 4ºC. Cells were then stained with biotinylated 9A11 mAb for 30 minutes at 4ºC, followed by streptavidin-APC (BD Pharmingen), and APC^+ cells were sorted using FACSVantage SE (Beckton Dickinson). The sorted cells were kept at 4˚C throughout the sorting procedure and then stained for various surface markers, as described above.

2.4.6 Immunostaining of NIH3T3 cells

Immunohistochemistry was performed to test the monoclonal antibody specificity on transfected NIH3T3 cells. Cells were plated on coverslips, which had been placed in 6 well plates ($2x10^5$ per well). The cells were fixed at RT in 2% paraformaledehyde (4% paraformaldehyde, 250mM HEPES, 6ml dH2O) for 20 minutes and quenched for 10 minutes at RT. Cells were permeabilized in 0.5% Triton X-100 (from Sigma) for 20 minutes at RT, washed three times with 0.1% PBS-Tween and blocked FACS block containing 0.5% saponin. The cells were stained with anti-HA.11 and/or 9A11 at 10μg/ml for 1 hour at RT and subsequently washed with 0.1% PBS-Tween to remove all unbound antibody. Cells were stained with donkey anti-mouse Cy3 (1/100 dilution, from Jackon) for 1 hour at RT and subsequently washed with 0.1% PBS-Tween. The cell nuclei were detected with DAPI (1:10,000 dilution, from Sigma). Coverslips were mounted with Vectashield (from Vector laboratories) and examined by conventional fluorescence microscopy on a Zeiss Axiovert 40. Images were processed using Adobe Photoshop version 6.0.

2.4.7 Endocytosis assays

For the endocytosis assays, transduced NIH3T3 or RAW264.7 cells were plated at $4x10^5$ cells/well and $5x10^5$ cells/well respectively, in 6 well plates the day before the experiment. At the start of the assay, cells were washed and then incubated in FACS block for 30 minutes at 4°C. Cells were stained with biotinylated 9A11 or anti-mDectin-1 (2A11; 10μg/ml) and incubated for 50 minutes at 4°C and subsequently washed with FACS wash, to remove unbound antibody. A cross-linking sheep anti-mouse antibody (5μg/ml, from Jackson) was added and the cells incubated for a further 30 min at 4°C. After washing in culture medium to remove unbound secondary antibody and azide, the cells were incubated in culture medium for the times indicated at either 37°C or 4°C. Cells were then stained with streptavidin-PE (from BD Pharmingen), and examined for remaining receptor surface expression by flow cytometery, as described above.

For microscopy, cells were plated at $2x10^5$/well on glass coverslips in 6-well plates the day before the experiment. Cells were treated the same as for the FACS based assay, only stained with 9A11 for 1 hour at 4°C and subsequently washed with FACS wash to remove unbound antibody. Cells were incubated for 30 minutes at either 37°C or 4°C in culture medium. After incubation the cells were fixed in 1ml fixative (4% paraformaldehyde; 250mM HEPES in dH_2O) for 20 minutes. The aldehyde groups were quenched with PBS Glycine (25mM Glycine powder in PBS) for 10 minutes and cells were permeabilized with 0.5% Triton X-100 for 20 minutes. Cells were washed with 0.1% PBS-Tween and permeabilised thereafter with 0.5% Saponin for 20 minutes. Cells were stained with LAMP-1 (clone ID4B; Developmental Studies Hybridoma Bank,

University of Iowa, Iowa City) for 1 hour at RT and washed with 0.1% PBS-Tween. Cells were then stained with donkey anti-mouse Cy3 and donkey anti-rat Alexa488 (1/100 dilution; both from Jackson) for 50 minutes at RT and subsequently washed with 0.1% PBS-Tween. Coverslips were mounted with Vectashield and examined by conventional fluorescence microscopy on a Zeiss Axiovert 40. Images were processed using Adobe Photoshop version 6.0.

2.4.8 Phagocytosis assays

To quantify phagocytosis by flow cytometry, transduced NIH3T3 fibroblasts or RAW264.7 macrophages were seeded at $2x10^5$cells/ well in 6 well plates the day before the experiment. When required, cells were pre-treated with 5μM cytochalasin D (from Calbiochem) for 40 min at 37˚C, to inhibit phagocytosis. Cells were then washed and FITC-labeled zymosan was added (1 particle/ cell) and allowed to bind for 1 hr at 4˚C. Unbound zymosan was removed by washing and the cells incubated at 37˚C for 2 hours (NIH3T3 cells) or 30 min (RAW264.7 cells) to allow particle uptake. The amount of internalized zymosan was then determined by flow cytometery, as previously described [51]. Briefly, external zymosan was stained with polyclonal rabbit anti-zymosan (from Invitrogen), which was subsequently detected with APC-labeled goat anti-rabbit antibody (from Invitrogen). Flow cytometeric analyses were performed by gating on the FITC-positive cell populations, which had bound zymosan, and the percentage of internalization was determined by comparing the APC-negative (internalized particles) versus the APC-positive (non-internalized particles) cell populations. The examination of phagocytosis by immunofluoresence microscopy was performed similarly, except that the

transduced cells were seeded at $3x10^4$ cells/ well on glass coverslips, 10 particles per cell of FITC-labeled zymosan were added, and the cells were allowed to internalize the particles for 45 min at 37˚C. Following this incubation, the cells were washed, fixed with 4% paraformaldehyde and permeabilised with 0.05% saponin in FACS block for 30 min at RT. Actin was stained with 1μM tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (from Sigma). Coverslips were mounted with Vectashield and examined by conventional fluorescence microscopy on a Zeiss Axiovert 40. Images were processed using Adobe Photoshop version 6.0.

2.4.9 Zymosan binding and cytokine assays

RAW264.7 and NIH3T3 transfectants were plated at $5x10^4$ cells/well in 24-well plates the day before the experiment. Cells were washed, soluble β-glucans (glucan phosphate; a kind gift from Dr. D. Williams, ETSU; 5μg/ml) added where appropriate, and the cells incubated for 20 minutes at 4˚C to allow inhibition of the Dectin-1 CRD [29]. Following the addition of FITC-labeled zymosan (25 particles/cell, from Invitrogen), cells were incubated at 4˚C for 60 minutes to allow binding, and then washed extensively to remove unbound particles. For the determination of TNF-α production, RAW264.7 cells were incubated for a further 3 hr and the amount of TNF-α released into the supernatants was determined by ELISA (from BD Biosciences). For measuring zymosan binding, cells were lysed in 3% Triton X-100 and the amount of bound zymosan was quantified by fluorometery using a Titertek Fluoroskan II (from Labsystems Group Ltd.).

For the analysis of the Syk-sufficient and Syk-deficient B-cells, $2x10^6$ transduced cells were stimulated with unlabelled zymosan (1 particle per cell, from Sigma) in 24 well suspension plates for 24 hours at 37˚C. IL-2 secreted into the supernatants was quantified by ELISA (from BD Biosciences). Where indicated, piceatennol (from Sigma) was included at 50µM.

2.4.10 Immunoprecipitations

$1x10^7$ RAW264.7 cells were stimulated with pervanadate for 1 min at 37ºC, and the cells lysed in ice-cold lysis buffer with protease inhibitors (from Roche). Nuclei and cell debris were removed by centrifugation, and the recovered supernatants were added to streptavidin beads (from Sigma), pre-coupled with biotinylated phosphorylated or unphosphorylated peptides (25µM). The CLEC9A peptides were generated commercially (from Sigma) and corresponded to the following region of the cytoplasmic tail of the receptor, ^{1}MHEEEIYRSLQWD^{13}. The Dectin-1 peptides have been described previously [120]. The beads were rotated for 2 hr at 4ºC and then washed, prior to analysis by Western blotting. Proteins in the immunoprecipitates were detected with anti-phosphotyrosine (clone 4G10), anti-Syk and anti-Lyn (from Santa Cruz), followed by appropriate HRP-linked secondary antibodies (from Jackson).

2.4.11 Reporter cells

Dr. E. Pyz (UCT) generated all the reporter chimeras consisting of the extracellular and transmembrane portions of mCLEC9A or Dectin-1 fused with the cytoplasmic tail of

CD3ξ chain were generated by PCR and cloned in the pMX-IP retroviral vector (a gift form Dr. T Kitamura, University of Tokyo, [186]) using the following primers:

	Forward primer	**Reverse primer**
mDectin-1	**5'**CCAACTAGTCCTTGGAGGCCCATTGCAGTGG**3'**	**5'**TTTGCGGCCGCTTACAGTTCCTTCTCACAGAT**3'**
mCLEC9A	**5'**AAAACTAGTGGAGCATGGTGTGTTGTGACG**3'**	**5'**AAAGCGGCCGCTCAGATGCAGGATCCAAAT**3'**
mCD3ξ	**5'**GTCTCGAGCCACCATGTTCAGCAGGAGTGCAG**3'**	**5'**CGATTCTAGAGTAGGCTTCTGCCATCTTGTC**3'**

All constructs were verified by sequencing, packaged into virons using HEK293T-based Phoenix ecotropic cells, described previously [74], and used to transduce BWZ.36 NFAT-LacZ cells.

2.4.12 Endogenous and exogenous ligand screen

C57/BL6 mice were obtained from the animal unit at the University of Cape Town. All animals were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. All procedures were performed in accordance with ethical guidelines established by the University of Cape Town.

For single cell suspensions, mouse organs were removed aseptically and disaggregated by maceration and strained through a 70μm cell filter. The cell suspension was washed twice and resuspended in culture medium. All steps were performed at 4°C.

For endogenous ligand screening, $1x10^5$ reporter cells were co-cultured with $1x10^6$ isolated mouse primary cells, for 20 hours in 48 well plates. For antibody cross-linking, 24 well plates were pre-coated with sheep anti-mouse IgG (50μg/ml) and followed by isotype or anti-mCLEC9A polyclonal antibody (10μg/ml). Following stimulation, IL-2 released into the supernatants was quantified by ELISA (from BD Pharmingen), and/or cells were stained for β-galactosidase expression.

For exogenous ligand screening, $1x10^5$ reporter cells were co-cultured with various heat-killed pathogens, for 20 hours in 48 well plates. Following stimulation, IL-2 released into the supernatants was quantified by ELISA, and/or cells were stained for β-galactosidase expression.

2.4.13 Immunohistochemistry

Mouse organs were collected, embedded and sectioned by Dr. V Tsoni (UCT). Cryosections were cut 5 to 10μm thick and placed on APES (from B&M Scientific) coated slides and stored at -20°C. Sections were left on the bench to reach RT and air dried before staining, this way the morphology is better preserved. The sections were washed with PBS, fixed in 4% paraformaldehyde for 10 minutes at RT and air dried before blocking in FACS block for 15 minutes at RT. Following the blocking step, sections were covered with 10μg/ml Fc-mCLEC9A and Fc-mCLEC12B and incubated at RT for 1 hour. Subsequently the sections were washed with PBS and stained with anti-human Cy3 (1/100 dilution, from Jackson) in the dark at RT for 30 minutes. The sections were washed extensively with PBS to remove all unbound antibody. The cell nuclei were

detected with DAPI. Coverslips were mounted with Vectashield and examined by conventional fluorescence microscopy on a Zeiss Axiovert 40. Images were processed using Adobe Photoshop version 6.0.

Chapter Three

Identification and *in silico* analysis

3.1 Identification of CLEC9A

Previous studies in our laboratory on the characterization of a CTLR called MICL led to the identification of a closely related receptor CLEC9A (accession number AY358265) based on sequence similarity and its genomic position [171]. The aim was to characterize this novel CTLR and determine the physiological role that it plays in the immune system.

In silico based analyses are often considered as the first step in the prediction of protein structure and function [187, 188]. The advances in computational methodologies have enhanced the comprehensive description of structural and functional domains or conserved motifs. Utilizing these computational assays has revealed several key features of the group V 'Dectin-1 cluster' of CTLRs. For example, all of these receptors consist of a single 'non-classical' CTLD, lacking the residues involved in Ca^{2+} -dependent binding; some contain cysteine residues in their stalk region essential for dimerization [12]; the cytoplasmic tail contain various conserved signaling motifs including ITAM-like [51, 101] and ITIMs [171, 179]; several other group V CTLRs also contain tri-acidic internalization motifs [33, 46].

This chapter describes the computational analysis of CLEC9A sequences used to analyze the primary and secondary structural components of this receptor, analyzing the homology between CLEC9A-related gene sequences found in other vertebrate genomes,

and determining possible functional similarities shared between related C-type lectins that are part of the 'Dectin-1 cluster. These *in silico* driven experiments revealed several features of CLEC9A, which will be discussed in this chapter.

3.2 Results

3.2.1 Sequence analysis and genomic structure of CLEC9A

The human *clec9A* gene is located on the plus strand of human chromosome 12 (locus 12p13) within the NKC, spanning approximately 13kb of genomic DNA (Figure 1.4). h*clec9A* consists of six exons, containing a single predicted open reading frame (ORF) encoding a protein of 241 amino acids with a predicted molecular mass of ~30kDa (Figure 3.1 A). The h*clec9A* gene is predicted to encode a type II transmembrane protein with a single extracellular CTLD, a stalk region and a cytoplasmic tail with a potential signaling motif (YxxL) [124] (Figure 3.1 B). The CTLD of hCLEC9A contains the six conserved cysteine residues likely to be involved in disulfide bond formation and stabilization of the CTLD fold [14], a typical feature of the group V CTLRs and lacks the residues involved in Ca^{2+}-dependend binding in classical C-type lectins [12]. This implies that hCLEC9A is part of the 'non-classical' CTLRs.

The transmembrane domain of human and murine hCLEC9A was characterized by submitting their amino acid sequences to the online database Pfam (Protein family database of alignments and HMMs)[e] and DNAMAN hydrophobicity plots (Figure 3.2 A).

[e] see website http://pfam.sanger.ac.uk/

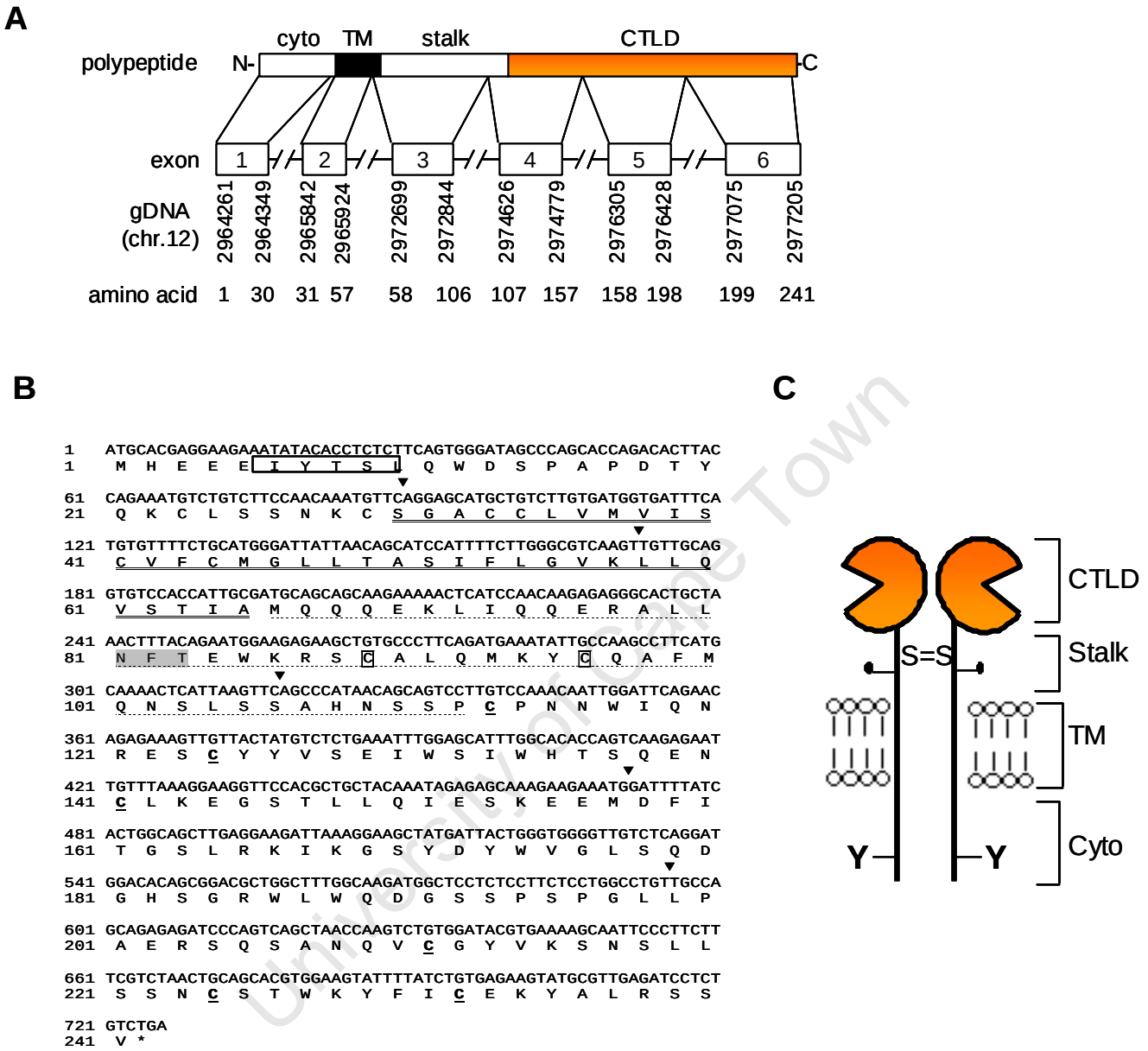
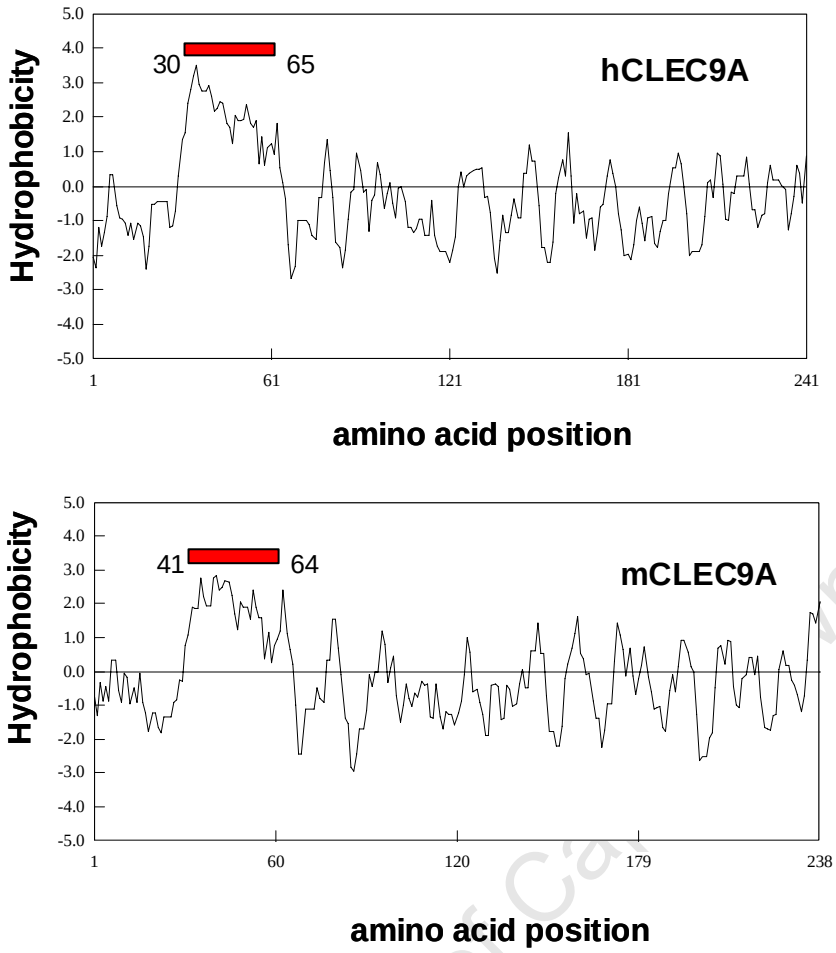


Fig. 3.1 Human CLEC9A gene structure and encoded polypeptide sequence. A) Schematic representation of the genomic structure of CLEC9A aligned with the encoded polypeptide, showing the positions of the exons in the genomic DNA (gDNA) and corresponding translated amino acid sequence. **B)** The nucleotide and translated protein sequence of CLEC9A. The putative ITAM-like motif is boxed; the transmembrane region is underlined by a double solid line; and the stalk region is underlined by a dashed line. The two cysteine residues in the stalk region are boxed and the six conserved cysteine residues in the CTLD are in bold and underlined. A putative N-glycosylation site is highlighted in grey. The black arrows (▼) indicate the exon boundaries of CLEC9A. **C)** Cartoon structure of predicted human CLEC9A (*S*: cysteine residue; *Y*: tyrosine residue; *CTLD*: cytoplasmic domain;*TM*: transmembrane domain; *Cyto*: cytoplasmic tail).

A



B

Sequence name	amino acid position	N-glycosylation potential	N-glycosylation result
hCLEC9A	81 NFTE	0.7237	Yes
hCLEC9A	109 NSSP	0.1581	No
hCLEC9A	223 NCST	0.4064	No
mCLEC9A	81 NLTQ	0.7811	Yes
mCLEC9A	133 NISK	0.5981	Yes

Fig. 3.2 Transmembrane and N-glycosylation predictions for CLEC9A. A) Hydrophobicity plots of human and murine CLEC9A. The red bars indicate the predicted amino acid position of the transmembrane domains. **B)** The putative N-glycosylation sites were predicted by using the online database NetNGlyc 1.0. Positive sites were selected based on the conserved amino acid sequences and their N-glycosylation potential.

N- and O-glycosylation sites were predicted by using the online database NetNGlyc 1.0 and NetOGlyc[f]. hCLEC9A consists of a single predicted N-linked glycosylation site (Figure 3.2 B), there were no predictions observed for any O-linked glycosylation sites, and possesses two cysteine residues in the stalk region, which may potentially lead to the dimerization of this molecule [12] (Figure 3.1 C). Additionally, h*clec9A* contains a putative internalization motif (tri-acidic sequence: *EEE*) in its cytoplasmic tail, indicating that this receptor may function as a potential endocytic receptor [33] (Figure 3.1 B).

The murine orthologue of human CLEC9A was identified based on its shared homology using the online database BLAST[e] and the genomic position. Sequence analysis indicates that the murine *clec9A* gene (accession number AK041288), like the human orthologue, encodes a type II transmembrane protein of 238 amino acids with a predicted molecular weight of ~30kDa (Figure 3.3 A). Similar to the human gene, m*clec9A* consists of six exons according to GenBank TM/EBI contig NT_039356 and is located on the plus strand of chromosome 6. The mCLEC9A retains most of the structural features as described for hCLEC9A, including the intracellular signaling motif (YxxL) (Figure 3.2 B). However, from the deduced amino acid sequence, the murine receptor appears to have an additional N-glycosylation site in its CTLD and lacks one of the cysteine residues in the stalk region (Figure 3.2B and 3.3 B).

[f] see website http:cbs.dtu.dk/services/NetNGlyc/ and /NetOGlyc/).
[e] see website http://blast.ncbi.nlm.nih.gov/Blast.cgi

A

```
1   ATGCATGCGGAAGAAATATATACCTCTCTTCAGTGGGACATTCCTACCTCAGAGGCCTCT
1    M  H  A  E  E  I  Y  T  S  L  Q  W  D  I  P  T  S  E  A  S

61  CAGAAGTGCCAATCCCCTAGCAAATGTTCAGGAGCATGGTGTGTTGTGACGATGATTTCC
21   Q  K  C  Q  S  P  S  K  C  S  G  A  W  C  V  V  T  M  I  S

121 TGTGTGGTCTGTATGGGCTTGTTAGCAACGTCCATTTTCTTGGGCATCAAGTTCTTCCAG
41   C  V  V  C  M  G  L  L  A  T  S  I  F  L  G  I  K  F  F  Q

181 GTATCCTCTCTTGTCTTGGAGCAGCAGGAAAGACTCATCCAACAGGACACAGCATTGGTG
61   V  S  S  L  V  L  E  Q  Q  E  R  L  I  Q  Q  D  T  A  L  V

241 AACCTTACACAGTGGCAGAGGAAATACACGCTGGAATACTGCCAAGCCTTACTGCAGAGA
81   N  L  T  Q  W  Q  R  K  Y  T  L  E  Y  C  Q  A  L  L  Q  R

301 TCTCTCCATTCAGGTAGTGACTGCAGCCCTTGTCCACACAACTGGATTCAGAATGGAAAA
101  S  L  H  S  G  S  D  C  S  P  C  P  H  N  W  I  Q  N  G  K

361 AGTTGTTACTATGTCTTTGAACGCTGGGAAATGTGGAACATCAGTAAGAAGAGCTGTTTA
121  S  C  Y  Y  V  F  E  R  W  E  M  W  N  I  S  K  K  S  C  L

421 AAAGAGGGCGCTAGTCTCTTTCAAATAGACAGCAAAGAAGAAATGGAGTTCATCAGCAGT
141  K  E  G  A  S  L  F  Q  I  D  S  K  E  E  M  E  F  I  S  S

481 ATAGGGAAACTCAAAGGAGGAAATAAATATTGGGTGGGAGTGTTTCAAGATGGAATCAGT
161  I  G  K  L  K  G  G  N  K  Y  W  V  G  V  F  Q  D  G  I  S

541 GGATCTTGGTTCTGGGAAGATGGCTCTTCTCCTCTCTCTGACTTGTTGCCAGCAGAAAGA
181  G  S  W  F  W  E  D  G  S  S  P  L  S  D  L  L  P  A  E  R

601 CAGCGATCAGCCGGCCAGATCTGTGGATACCTCAAAGATTCTACTCTCATCTCAGATAAG
201  Q  R  S  A  G  Q  I  C  G  Y  L  K  D  S  T  L  I  S  D  K

661 TGCGATAGCTGGAAATATTTTATCTGTGAGAAGAAGGCATTTGGATCCTGCATCTGA
221  C  D  S  W  K  Y  F  I  C  E  K  K  A  F  G  S  C  I  *
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B

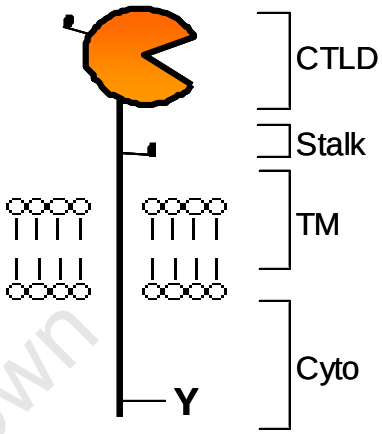


Fig. 3.3 Murine CLEC9A gene structure. A) The nucleotide and translated protein sequence of murine CLEC9A. The putative signaling motif is boxed; the transmembrane region is underlined by a double solid line; and the stalk region is underlined by a dashed line. The single cysteine residue in the stalk region is boxed and the conserved cysteine residues in the CTLD are in bold and underlined. The black arrows (▼) indicate the exon boundaries of mCLEC9A. **B)** Cartoon structure of the predicted mCLEC9A receptor (*Y*: tyrosine residue; *CTLD*: cytoplasmic domain; *TM*: transmembrane domain; *Cyto*: cytoplasmic tail).

3.2.2 Homologies with other species and known C-type lectins

The predicted hCLEC9A amino acid sequence was used to identify its closest homologues by searching various on-line databases[g]. As indicated by sequence analysis mCLEC9A is 53% identical to hCLEC9A, and retains most of the structural features described previously, including the potential intracellular signaling motif [124] (Figure 3.4). CLEC9A-related gene sequences were found in other vertebrate genomes, including *Macaca mulatta, Canis familiaris, Bos taurus* and *Rattus norvegicus* (Figure 3.5). There is a 77.94% similarity shared between the protein sequences of the different CLEC9A species. The six conserved cysteine residues in the CTLD, the signaling motif in the cytoplasmic tail and one of the predicted N-linked glycosylation sites were retained among all gene sequences as indicated in Figure 3.5. All the CLEC9A-related genes had only a single cysteine residue present in the stalk region, except for the human gene, which possesses two (Figure 3.5).

Previously published analysis suggests that the CTLD of CLEC9A is closely related to the one found in the killer-lectin receptor 1 (KLR-I) [189]. Our analysis, using NCBI Blast protein analysis[g], shows that despite having a high degree of similarity to KLR-I and some members of the KLR complex, hCLEC9A is phylogenetically most related to MICL (CLEC12A) and CLEC12B, whereas the mCLEC9A shares most sequence similarity with mDectin-1 (CLEC7A) as well as mCLEC12B. Thus CLEC9A is mostly homologous to other C-type lectin-like molecules found on the 'Dectin-1 cluster' of CTLRs (Figure 3.6 A).

[g] http://www.ncbi.nlm.nih.gov/BLAST/

Fig. 3.4 Sequence homology between human and murine CLEC9A. Comparison of the human and murine CLEC9A polypeptide sequences. Light gray highlights indicate identity and dark gray highlights indicates similarity. The signaling motif in the cytoplasmic tail is boxed (YxxL). The missing cysteine in the stalk region of mCLEC9A is indicated with an asterisk (*).

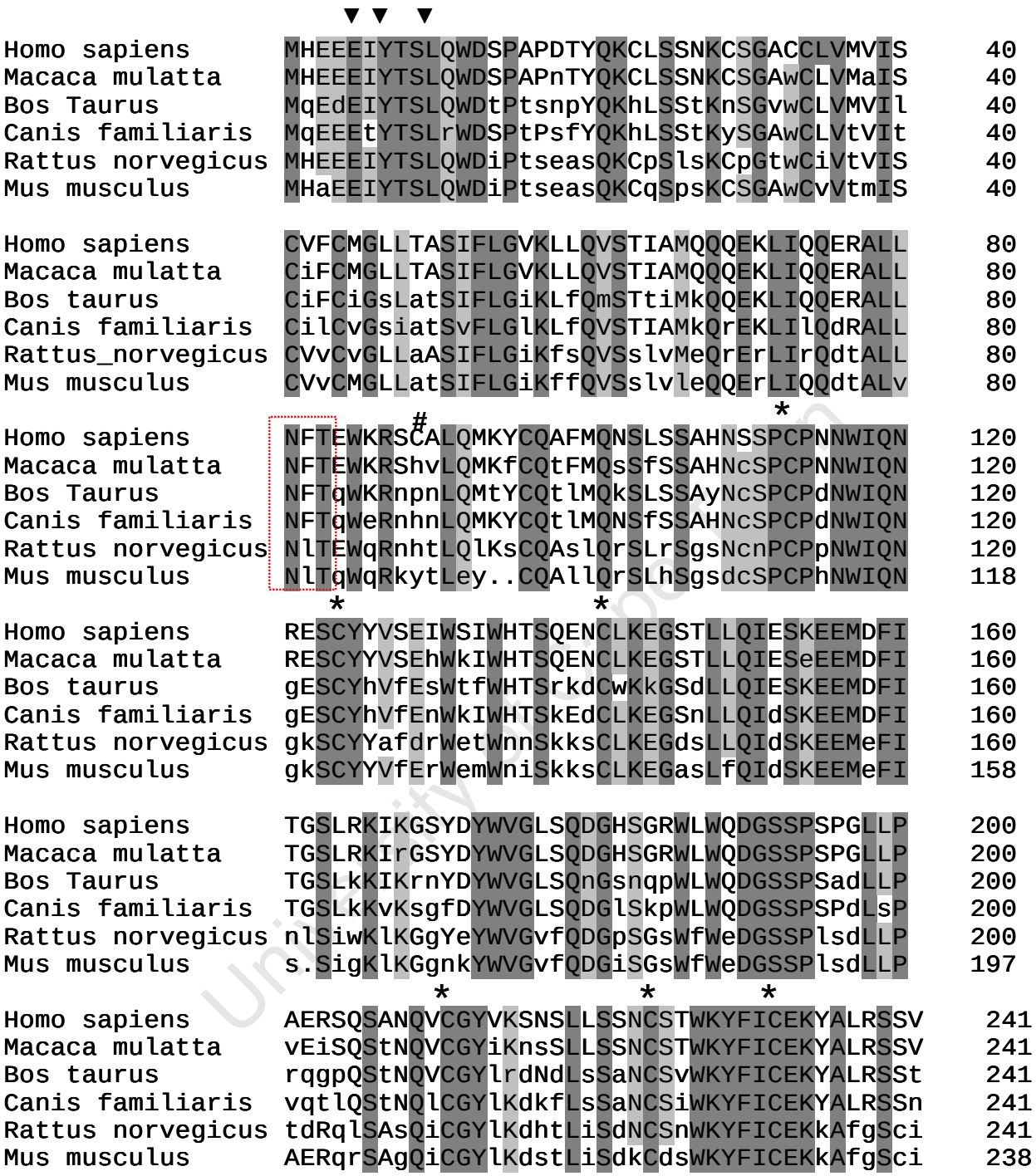
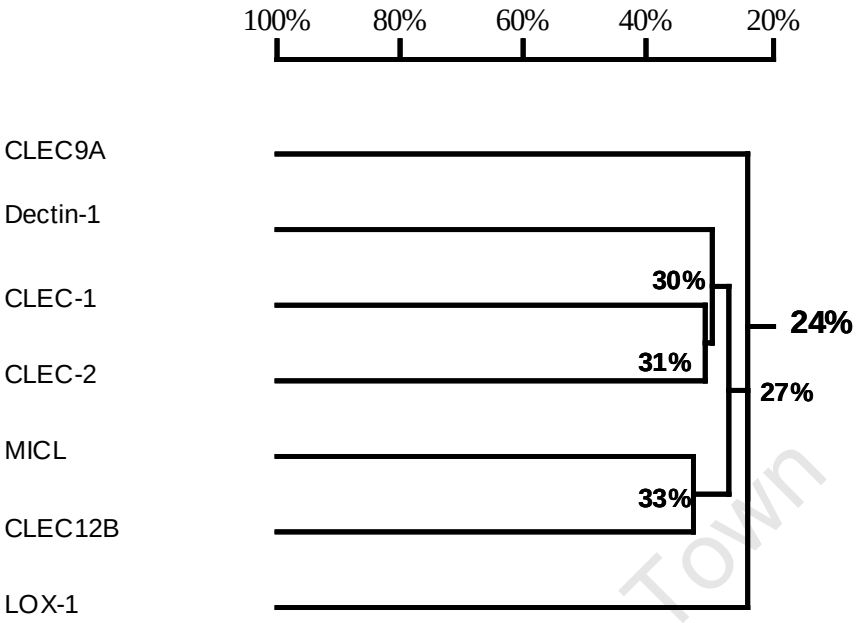


Fig. 3.5 Homology shared between CLEC9A related genes sequences found in other vertebrate genomes. The full length CLEC9A protein sequences from *Homo sapiens*, *Macaca mulatta*, *Canis familiaris*, *Bos taurus*, *Mus musculus* and *Rattus norvegicus* share 77.94% identity. Homology is indicated by dark gray (100%) and light gray (>75%). The signaling motif in the cytoplasmic tail (ExYxxL) is retained and conserved between species as indicated by the black arrows (▼). The conserved six cysteine residues involved in the CTLD fold are indicated by the asteriks (*). The extra cysteine residue in the stalk region of hCLEC9A is indicated by (#) and the predicted N-glycosylation sites are boxed (NF/LT).

A



B

```
                        *  *  *
hCLECA          ........MHEEEIYTSLQWDSPAPDTYQKCLSSNKCS       30
mCLEC9A         ........MHaEEIYTSLQWDiPtseasQKCqSpsKCS       30
Dectin-1        meyhpdlenldEdgYTqLhfDSqsntriav........      30
CLEC-2          ........MqdEdgYitLniktrkPalvsvgpaSssww       30
Consensus               e  y  l
```


<u>Fig. 3.6</u> Comparative homology of hCLEC9A with receptors that are part of the 'Dectin-1 cluster' of CTLRs and the ITAM-bearing receptors. A) The phylogenetic tree represents the homology shared by the full length human protein sequences of CLEC9A and the related "Dectin-1 cluster' of CTLRs. Dectin-1 (NP_922938); CLEC-1(NP_057595); CLEC-2 (NP_057593); MICL (NT_009714); CLEC12B (NP_995324) and LOX-1 (NCBI XP_149798.2). The values indicate percentage similarity of each CTLRs to the hCLEC9A. **B)** Alignment of the cytoplasmic tail of CLEC9A with that of Dectin-1[50] and CLEC-2[127] showing the first 30 residues of the ITAM-containing signaling motifs. The conserved residues of the ITAMs are indicated by asterisks (*); the full consensus sequence ExYxxL. Light gray indicates 100% identity and dark gray >50% identity.

From this alignment CLEC9A possesses a tyrosine residue in its cytoplasmic tail that resembles the ITAM-like motif of Dectin-1 [51] and CLEC-2 [125] (Figure 3.6 B), suggesting that CLEC9A may function as a potential activatory receptor. The cytoplasmic domain of most CTLRs is essential for their physiological role in the immune system [101, 103].

3.3 Discussion

Using *in silico* based analysis I characterized a novel CTLR, called CLEC9A. This chapter describes the genomic, mRNA and predicted amino acid sequences for CLEC9A, including sequence based domain and motif analysis, the identification of shared homologues and predicted function. CLEC9A is part of the group V CTLRs, in terms of exon and polypeptide structures, as well as a CTLD that conforms to the basic structure of C-type lectins but lacks the conserved Ca^{2+}-binding residues involved in carbohydrate binding (Figure 3.1 B). Subsequently two other groups (Caminschi *et al.* [190] and Sancho *et al.* [191]) also identified CLEC9A as a novel C-type lectin receptor, showing that human and murine CLEC9A genes each encode a type II transmembrane protein with a single extracellular CTLD lacking the Ca^{2+}-binding residues involved in carbohydrate binding as well as a highly conserved tyrosine residue in the cytoplasmic tail. Thus their results confirm our findings.

The human and murine CLEC9A sequences possess a tyrosine residue in their respective cytoplasmic tails within a potential signaling motif (YxxL) [124] (Figure 3.2 B). All the 'Dectin-1 cluster' receptors contain conserved activatory/inhibitory signaling motifs,

except LOX-1, that trigger downstream signaling cascades which regulate various functions in immune cells [78]. The tyrosine residue in the cytoplasmic tail of hCLEC9A resembles the ITAM-like motif of Dectin-1 [51] and CLEC-2 [125] suggesting that CLEC9A might function as a possible activatory receptor. The ITAM-like motif of Dectin-1 is capable of triggering various cellular responses including phagocytosis, synthesis of ROIs and cytokine production [106, 119, 120]. Recently it was shown that Dectin-1 can use this motif to couple Syk kinase and that agonists of Dectin-1 signaling through Syk promote the activation of DCs [103, 106, 120]. This suggests that CLEC9A has the potential to induce similar cellular responses, with regards to its putative activation motif that resembles the ITAM-like motif of Dectin-1.

C-type lectins that contain tri-acidic internalization motifs (DEC-205, DC-SIGN, BDCA-2, Dectin-1 and CLEC-1) target internalized antigens to the lysosomes and MHC class II^+ late endosomes [41, 192] and function as antigen-uptake receptors [33]. Sequence analysis of CLEC9A shows that this receptor contains a tri-acidic motif in its cytoplasmic tail (Figure 3.2 B), indicating that this receptor may be able to function as an endocytic receptor and have the potential to act as an antigen-uptake receptor. Additionally, hCLEC9A possess two extra cytsteine residues in its stalk region that could allow protein homodi- or trimerization [12], whereas, the murine orthologue of CLEC9A show the presence of a single cysteine residue. These oligomers may be important for ligand binding. Conversely, the two other groups identified and characterized a seven exon version of mCLEC9A (accession AKO36399.1) encoding a protein of 264 amino acids [190, 191]. The ‘extra’ exon is located in the CTLD of mCLEC9A, although this

transcript retains most of the structural features of the six exon mCLEC9A, there might be some functional differences observed between the two transcripts, which will be discussed in chapter 4.

The CLEC9A gene is highly conserved between related gene sequences found on different vertebrate genomes as indicated in Figure 3.5. This suggests that the function of this receptor may have been retained through evolution, because of the 77.94% similarity shared between species as well as the conserved signaling motif in the cytoplasmic tail.

In conclusion, the predictions made in this chapter confirmed that CLEC9A is a novel CTLRs that is part of the group V 'Dectin-1 cluster' and shares various structural features with the members of this cluster. Our predictions from this analysis are that CLEC9A may be expressed as a glycosylated homodimer, have the ability to function as an activation receptor with the additional potential to mediate endocytosis. The expression, regulation and function of CLEC9A will be presented in chapters 4, 5 and 6 along with future investigations that will be outlined in chapter 7.

Chapter Four

Characterization of CLEC9A expression

4.1 Introduction

The aim of this chapter was to characterize the protein and cellular expression of CLEC9A. Sequence analyses indicate that CLEC9A is most homologous to the group V 'Dectin-1 cluster' of CTLRs. A characteristic feature of the group V receptors is the presence of cysteine residues within their stalk region, which is involved in homo- or hetero-dimerization (http://ctld.glycob.ox.ac.uk/). This may be a possible way to increase the efficiency of ligand binding [193]. hCLEC9A contains two cysteine residues in its stalk region, which may therefore lead to dimerization and facilitate ligand binding.

Establishing the cellular distribution of a cell surface receptor is important to try and understand its function. Many of the 'Dectin-1 cluster' receptors are expressed on myeloid cells and/or endothelium and possess activating or inhibitory signaling motifs that trigger downstream signaling events that regulate the role of these cells [74, 144, 162, 171]. Dectin-1, for example is known to be expressed on macrophages and neutrophils, where some of its major functions lie, including phagocytosis, the production of ROS and the induction of inflammatory cytokines [102, 121].

In this chapter I wanted to characterize the protein expression of CLEC9A in tissues, cell lines and its expression profile on primary cells.

4.2 Results

4.2.1 Analysis of CLEC9A expression at RNA level

The first step in analyzing the expression of human CLEC9A was to probe various tissues for the presence of CLEC9A mRNA. The mRNA expression was examined by RT-PCR, using primers that specifically amplified the entire ORF of hCLEC9A from cDNA isolated from a variety of human tissues (Figure 4.1). hCLEC9A expression was found in most organs, but predominately in brain, thymus and spleen. Its expression in human tissues was detected as a single band at the predicted size of 0.8kb, suggesting that there were no alternative splice variants. A water sample was included as a negative control and a plasmid containing the hCLEC9A sequence (accession number AY358265) was used as a positive control (Figure 4.1). Additionally, using specific primers, the human housekeeping gene G3PDH was included as a loading control showing that the levels of expression between all tissues were comparable, indicating that the cDNA used was of a good quality (Figure 4.1 lower panel).

The mRNA expression of murine CLEC9A was also examined by RT-PCR, using primers that specifically amplifed the entire ORF from cDNA isolated from a variety of mouse tissues. RT-PCR analysis revealed that mCLEC9A was detected in most tissues as multiple bands, which upon sequencing, were shown to be due to alternative splicing (Figure 4.2 A). High levels of mCLEC9A expression were observed in heart, spleen, skeletal muscle and day 17 embryo, and

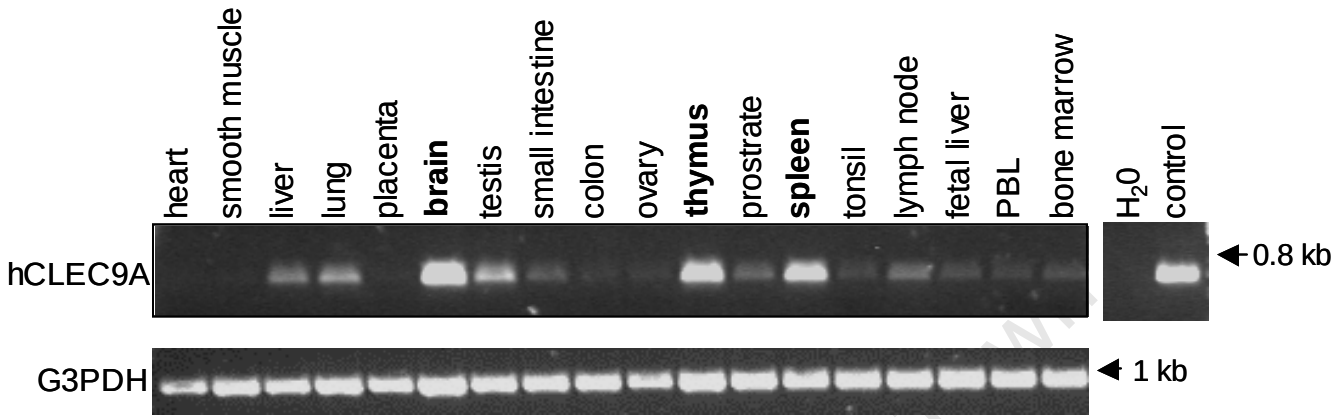
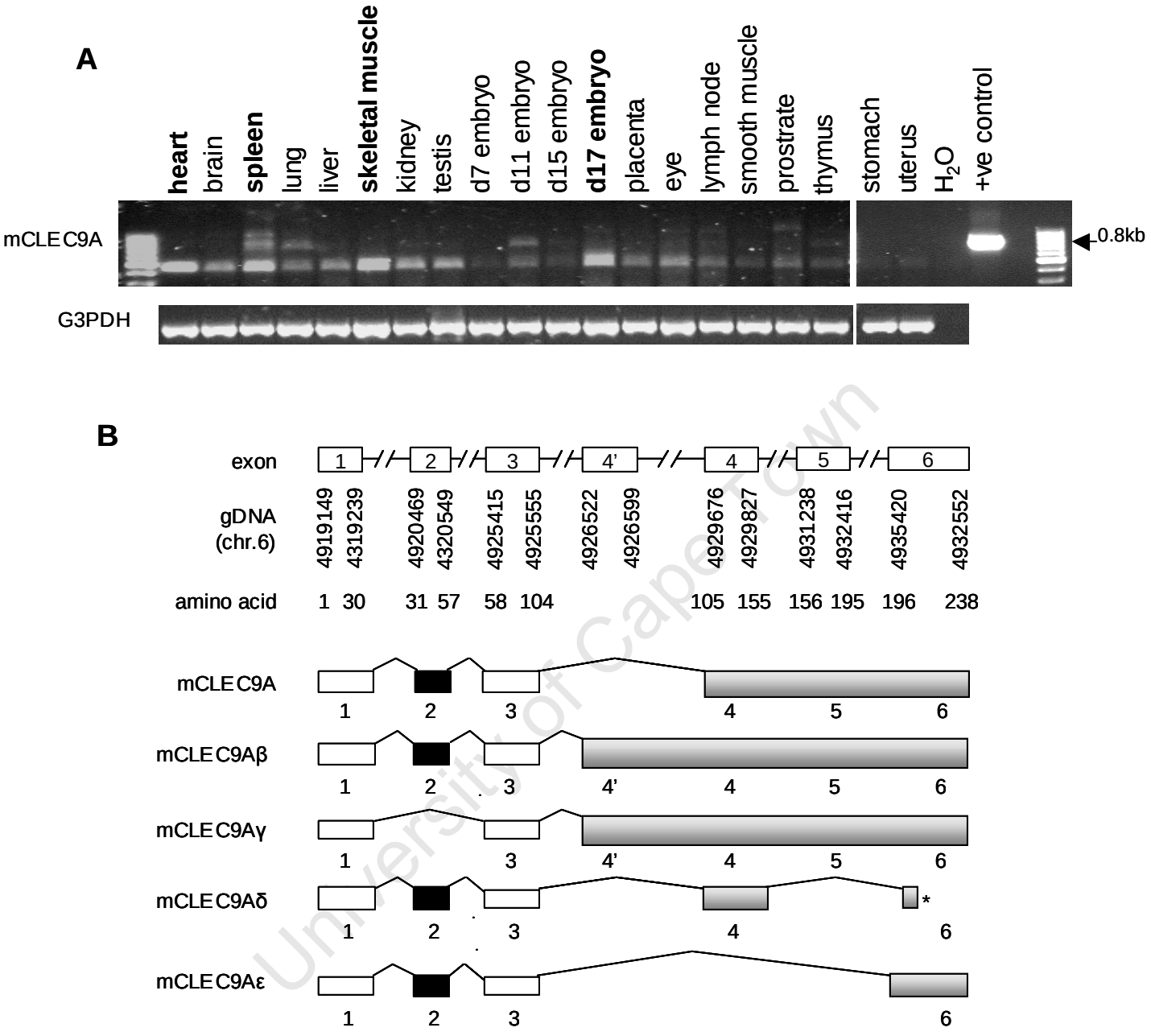


Fig.4.1 Distribution of human CLEC9A transcripts and schematic representation of the constructs used in this study. Various human tissues were subjected to RT-PCR using hCLEC9A specific primers (upper panel) or G3PDH primers (lower panel). A water control was included as a negative control and a plasmid containing the hCLEC9A sequence (accession number AY358265) was included as a positive control. hCLEC9A is expressed in most tissues, but predominantly in the brain, spleen and thymus.



<u>Fig. 4.2</u> mCLEC9A is expressed in multiple tissues as a number of alternatively spliced isoforms. A) RT- PCR analysis showing expression of mCLEC9A, detected as multiple bands, in most tissues. mCLEC9A specific primers (upper panel) or G3PDH primers (lower panel) as well as a water control was included as a negative control. A plasmid containing the mCLEC9A sequence (accession number AK041288) represented the positive control. **B)** Schematic representation of the genomic structure of CLEC9A aligned with the predicted polypeptides, generated by alternative splicing, as indicated. The positions of the exons in the genomic DNA (gDNA) and corresponding translated amino acid sequence of full-length mCLEC9A are shown. The asterisk indicates a premature stop codon.

intermediate levels in brain, lung, liver, kidney, testis and eye. The isoforms of mCLEC9A were predominantly present in spleen, lung and day 11 embryo. A water sample was included as a negative control and a plasmid containing the mCLEC9A sequence (accession number AK041288) represented the positive control (Figure 4.2 A upper panel). The murine housekeeping gene G3PDH was also included as a loading control, using primers specific for this gene, showing similar levels of expression in all tissues and indicating that the cDNA was of a good quality (Fig 4.2 A lower panel).

The genomic structure of mCLEC9A aligned with the various predicted polypeptides, generated by alternative splicing, and the missing exons of each isoform are schematically represented in Figure 4.2 B.

4.2.2 CLEC9A is expressed at the cell surface as a glycosylated dimer.

To characterize the protein expression of human and murine CLEC9A, we cloned the full-length hCLEC9A (cDNA obtained from peripheral mononuclear cells) and mCLEC9A (cDNA obtained from spleen) in order to perform transfection studies. Receptor constructs containing a C-terminal epitope HA-tag were used initially, since anti-CLEC9A antibodies were not available at the time. Dectin-1 was included as a control because of its homology to CLEC9A, and the great amount of data that has been generated in our laboratory regarding its characterization and we have the necessary reagents. Cartoon structures of the constructs generated are illustrated in Figure 4.3.

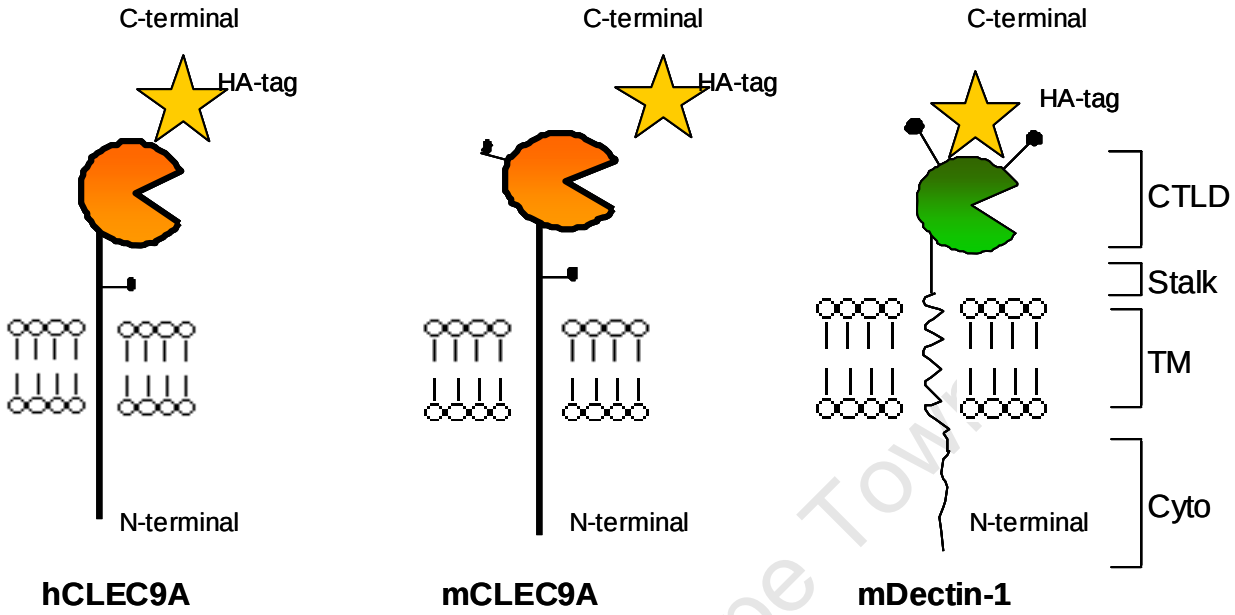


Fig. 4.3 Schematic representation of constructs used in CLEC9A transfectants. Cartoon structures of wild type full-length hCLEC9A, wild type full-length mCLEC9A and wild type full-length Dectin-1 showing a single C-type lectin-like domain (CTLD), a stalk region, a single transmembrane domain (TM) and a cytoplasmic tail (Cyto), respectively. Predicted N-glycosylation sites are indicated by lollipop structures and the stars indicate the positioning of HA epitope tags.

To investigate whether CLEC9A was expressed and transported to the cell surface in heterologous cells, live NIH3T3 fibroblasts were retroviraly transduced with a HA-tagged full-length human and murine CLEC9A were stained with an anti-HA antibody. Flow cytometry analysis indicated that both human and murine CLEC9A was expressed and transported to the cell surface in heterologous cells (Figure 4.4 A).

Sequence analysis indicated that hCLEC9A possess two cysteine residues in its stalk region, which may lead to dimerization, and both human and murine CLEC9A have potential N-glycosylation sites (Figure 3.2 B). To confirm these predictions immunoprecipitations of HA-tagged CLEC9A transfected cells were analysed by Western blot. Probbing with anti-HA antibody under nonreduced conditions showed that the majority of hCLEC9A protein is expressed as a dimer with a molecular size of around 97kDa, corresponding to twice the predicted molecular mass (Figure 4.4 B). The protein bands were reduced to two bands of approximately 50 and 55kDa under reduced conditions.

This confirmed that hCLEC9A is expressed as a homodimer as it was predicted for sequence analysis. Western blot analysis shows that mCLEC9A was expressed as a monomer (Figure 4.4 B) with a MW of approximately 35kDa under nonreduced and reduced conditions. This may be due to the presence of only a single cysteine residue in the stalk region that is unable to form a disulphide bond that is required for homodimerization. Control cells expressing Dectin-1, showed that this receptor is

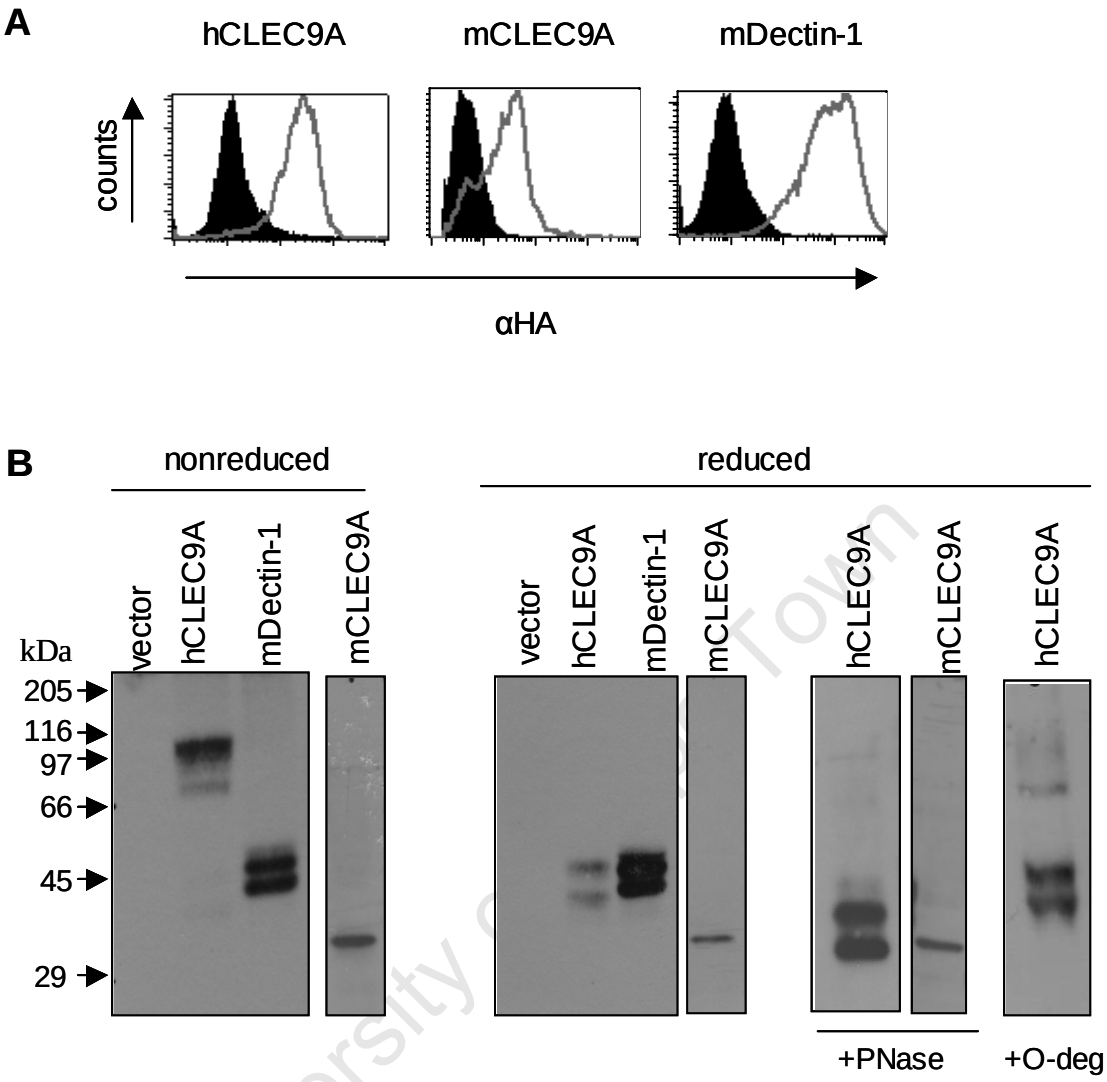


Fig. 4.4 Expression of hCLEC9A as a glycosylated dimer and mCLEC9A as a monomer. **A)** Flow cytometry of live NIH3T3 cells stably expressing HA-tagged hCLEC9A, mCLEC9A or mDectin-1, as indicated (grey lines), demonstrating expression of these receptors at the cell surface. The filled histograms indicate vector only transduced controls. **B)** Anti-HA Western blotting of lysates from these cells demonstrating that CLEC9A is expressed as a dimer of ~100kDa under nonreducing conditions, which can be resolved to two bands of ~45 and 50kDa under reducing conditions. mCLEC9A is expressed as single band close to the predicted MW of ~30kD, indicating that mCLEC9 is expressed as a monomer. Treatment of the cell lysates with PNase, which removed N-linked glycosylation, reduced the molecular weight (MW) of the bands in hCLEC9a to close to that expected from the predicted amino acid sequence, indicating that hCLEC9A is N-glycosylated. Treatment with PNase did not alter the MW of mCLEC9A indicating that mCLEC9A is not N-glycosylated. The post-translational modification giving rise to the second band seen in these lysates, was not due to O-glycosylation, but was also seen with mDectin-1, which is expressed as a monomer and was included as a control for these experiments. Lysates from vector-only transfectants were included as a negative control.

expressed as a monomer (Figure 4.4 B), as expected [102]. Vector-only transfected cells were included as a negative control.

To confirm whether the high MW of hCLEC9A under reduced conditions was due to glycosylation, cell lysates from NIH3T3 cells transfected with HA-tagged CLEC9A were N- and O- deglycosylated by treatment with PNase and O-glycosidase respectively. The two apparent bands were reduced to a MW of approximately 30 and 35kDa after PNase treatment (Figure 4.4 B), which removed N-linked glycosylation, indicating that hCLEC9A was glycosylated. Treatment with PNase did not alter the MW of mCLEC9A, indicating that mCLEC9A is not N-glycosylated. The post-translational modification giving rise to the second band apparent in the cell lysates of hCLEC9A was unlikely due to O-glycosylation, since there was no reduction in the MW after treatment with this O-glycosidase treatment (Figure 4.4 B). The presence of a second band in these cell lysates, which was also observed with Dectin-1, may be due to other possible posttranslational modifications.

4.2.3 Expression of hCLEC9A on primary cells

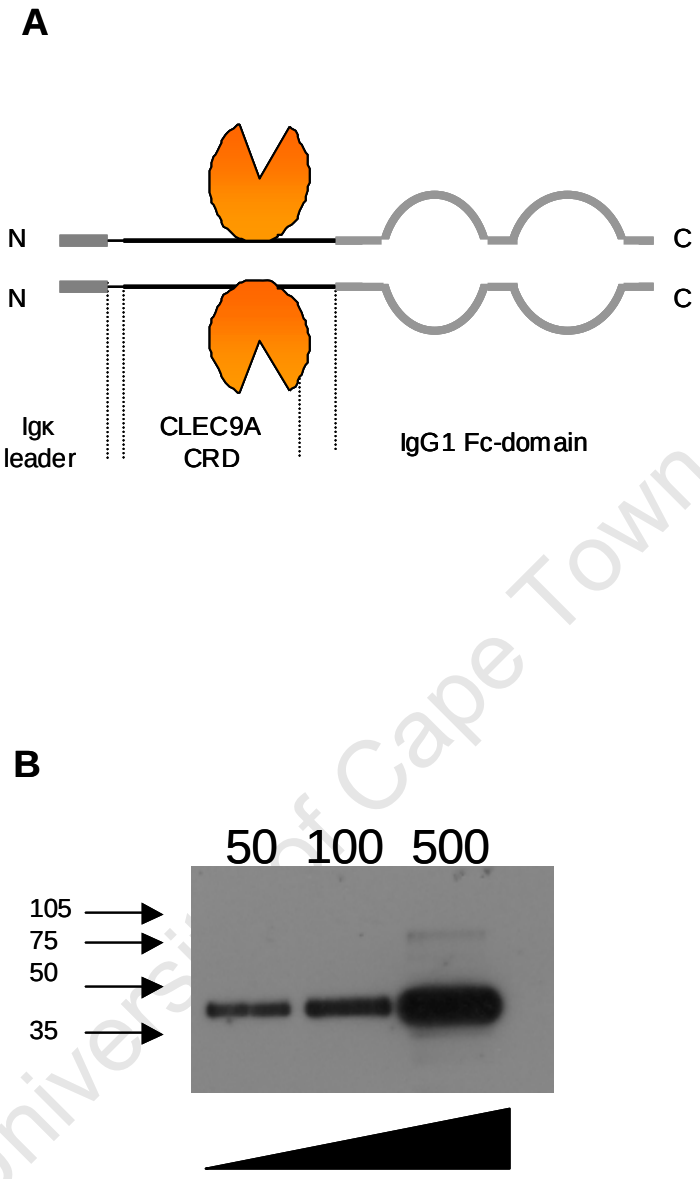
The mRNA expression of hCLEC9A gave helpful clues as to the *in vivo* distribution of CLEC9A protein. However, the presence of a particular mRNA does not necessarily correlate with the expression of its encoded protein [194, 195]. Monoclonal antibodies (mAbs) are also a useful tool for expression and functional assays. Therefore, I generated a novel mAb to characterize the cellular distribution of hCLEC9A in more detail, which was the initial focus. We also attempted to the generation of a mAb against mCLEC9A,

which was initially unsuccessful, however, the production of a novel mAb against mCLEC9A is currently in progress.

4.2.4 The generation and testing of anti-CLEC9A antibody

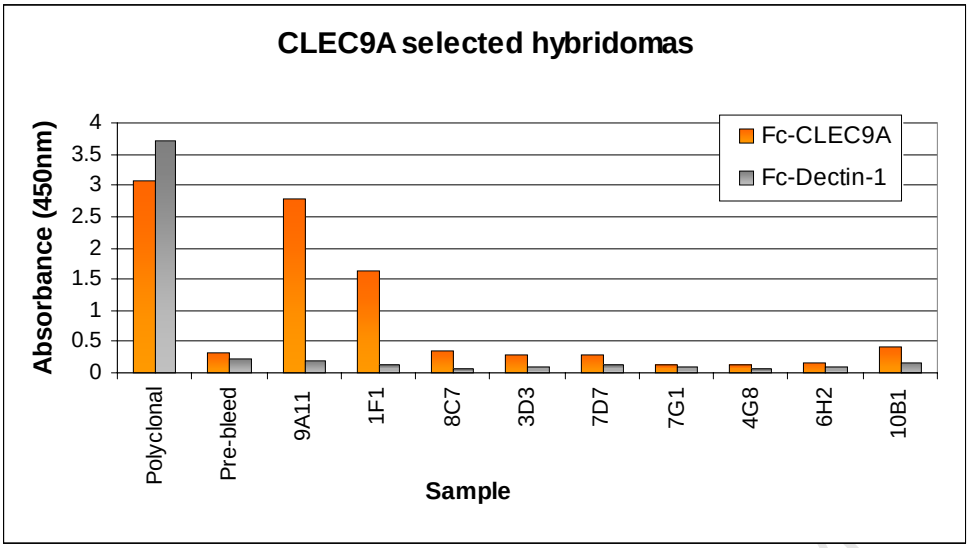
To generate a monoclonal antibody against hCLEC9A, a soluble Fc-CLEC9A fusion protein was generated, comprising of the CTLD of hCLEC9A fused with a human IgG1 Fc domain, separated with a short spacer (Figure 4.5 A). The Fc domain allowed for the isolation of a highly purified protein by protein A affinity chromatography. The purified protein has a MW of approximately 40kDa under reduced conditions (Figure 4.5 B). There is no apparent N-glycosylation of the soluble Fc-CLEC9A protein, because the predicted N-glycosylation site in the stalk region was deleted upon the generation of the soluble Fc-protein.

To generate the anti-CLEC9A antibody, mice were immunized with the purified Fc-CLEC9A antigen and hybridomas were generated according to standard protocols (see *Materials and Methods*). The hybridoma supernatants were screened by ELISA and those that were positive for Fc-CLEC9A were examined for functionality in other assays. A similarly designed control Fc-protein (Fc-Dectin-1) was included in both ELISA and Western blot assays to demonstrate anti-CLEC9A ecto-domain specificity rather than anti-Fc specificity (Figure 4.6 A). Of the 996 supernatants tested only 11 hybridomas demonstrated specificity for Fc-CLEC9A. These hybridomas were expanded and clonally diluted. Thereafter the supernatants were analyzed again for their reactivity against hCLEC9A. Only two hybridomas (9A11 and 10B1) tested positive under nonreducing



<u>Fig. 4.5</u> Generating a soluble Fc fusion protein for CLEC9A. A) Cartoon structure of the CLEC9A Fc fusion protein used for the generation of a monoclonal antibody against CLEC9A. **B)** Western blot showing a titration series of Fc-CLEC9A 50, 100, 500 ng protein with a predicted MW of approximately 40kDa under reduced conditions.

A



B

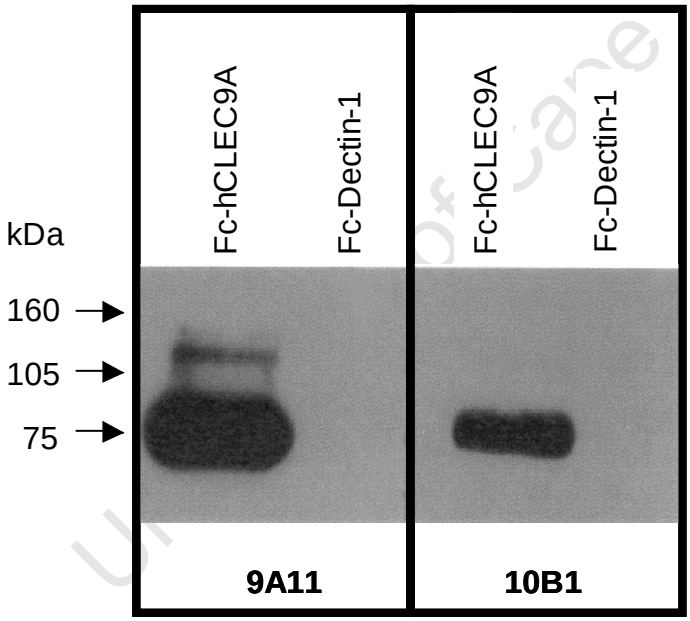


Fig. 4.6 Generation of mouse anti-human CLEC9A mAb. The supernatants of various hybridomas were screened by **A)** ELISA and **B)** Western blot under nonreduced conditions. Dectin-1 was included as a control to confirm hybridoma specificity for the CTLD of CLEC9A. 9A11 and 10B1 were the two hybridomas that were consistently positive. The 1F1 hybridoma was not functional in Western blot assays. The serum from mice before immunization (pre-bleed) was used as a negative control and serum after immunization (polyclonal) was included as a positive control.

conditions by Western blot (Figure 4.6 B). The 9 remaining hybridomas were not functional by Western blot. The 10B1 hybridoma tested negative by flow cytometry. The 9A11 hybridoma was the only hybridoma that tested positive with ELISA, flow cytometry and Western blot, although it did not recognize hCLEC9A under reducing conditions. This hybridoma was clonally diluted three times and the cells expanded for antibody purification.

To demonstrate that the purified 9A11 (IgG1) mAb generated against Fc-hCLEC9A was specific for detecting hCLEC9A, I utilized various hCLEC9A-containing reagents. NIH3T3- HA-tagged CLEC9A transduced cells, which confirmed surface expression of CLEC9A (as shown before in Figure 4.4A), were stained with 9A11 and tested positive by flow cytometry, but not the vector control cells; this reactivity was not significantly affected by fixation (Figure 4.7 A).

Western blot analysis of lysates from NIH3T3 transfected with HA-tagged hCLEC9A, showed that 9A11, under nonreducing conditions, specifically recognized hCLEC9A (Figure 4.7 B). The range of protein sizes, resolving at 66-97 kDa, was similar to the results obtained previously in Figure 4.4 B. These sizes were comparable when the membranes were probed with an anti-HA mAb (Figure 4.7 B). As stated before the 9A11 antibody did not detect any hCLEC9A protein under reducing conditions (data not shown).

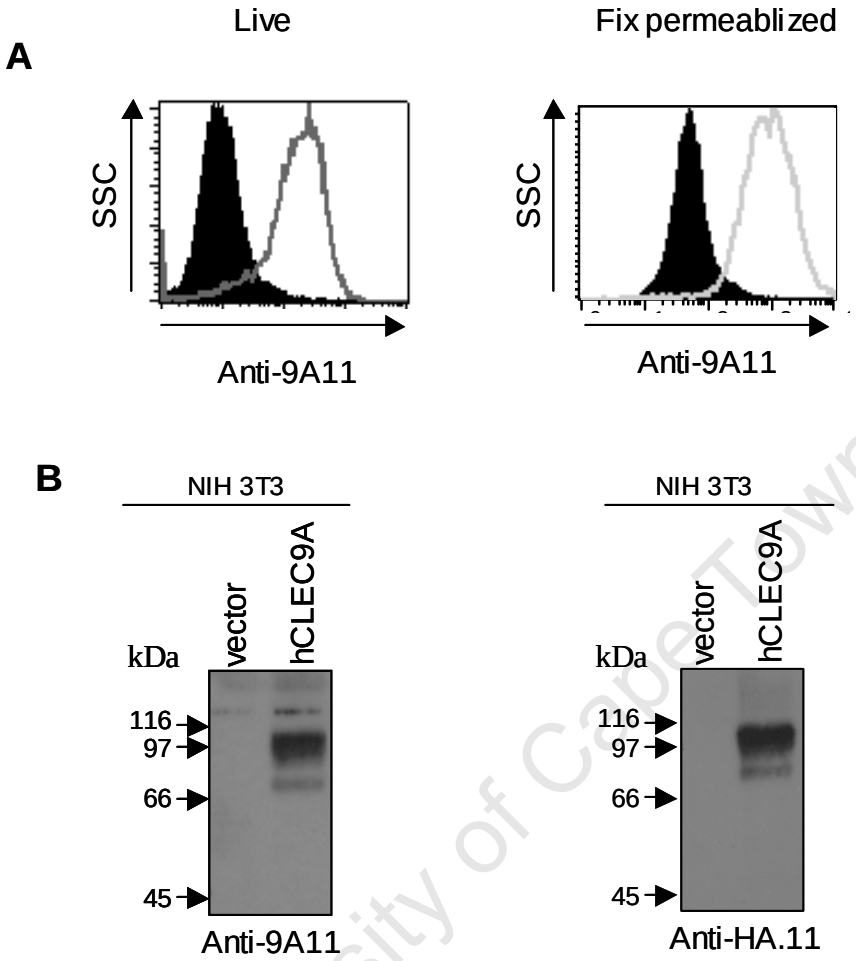
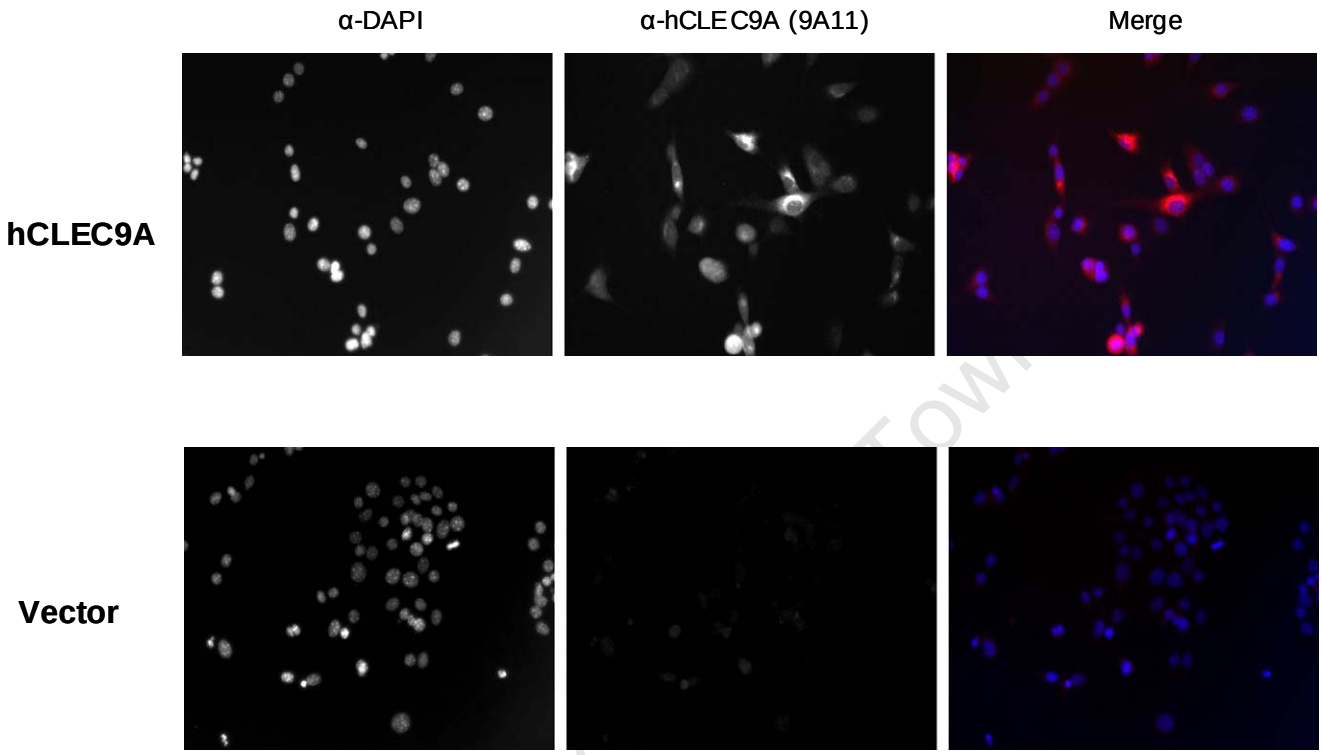


Fig. 4.7 Confirming anti-CLEC9A mAb specificity. A) By flow cytometry, 9A11 reacted with live and fixed permeabilized NIH3T3- CLEC9A transduced cells (grey lines). The filled histograms indicate vector-only transduced controls. **B)** In Western blotting, under nonreducing conditions, 9A11 specifically recognized a range of protein sizes, resolving at 66-97 kDa, from lysates of NIH3T3 CLEC9A transduced cells. Anti-HA.11 staining on RAW 264.7- CLEC9A transduced cells was included as a positive control.



<u>Fig. 4.8</u> Immunostainning of transduced cells with anti-CLEC9A (9A11). Immunohistochemistry of NIH3T3- CLEC9A transduced cells (upper lane) stained with 9A11 (red) and nuclear staining with DAPI (blue). Vector-control cells were included to demonstrate the specificity of 9A11 (lower lane).

Finally, 9A11 was confirmed to be specific for hCLEC9A by immunocytochemistry, in paraformaldehyde-fixed NIH3T3 transfectants (Figure 4.8). A vector-only control was included as a negative control. However, when human tissues (thymus and kidney) were paraformaldehyde-fixed and stained with 9A11, we did not detect hCLEC9A expression (data not shown) as it was expected in the PCR results where hCLEC9A mRNA was predominantly expressed. The reason for this could be that the protein expression of hCLEC9A is absent in these tissues, low cell number expressing hCLEC9A or undetectable by 9A11. We were unable to test any other tissues due to the difficulty in obtaining ethical approval for human tissue samples.

4.2.5 Distribution of CLEC9A within peripheral blood

The mRNA results showed that hCLEC9A was transcribed in peripheral blood leukocytes (PBLs) (Figure 4.1), indicating that hCLEC9A may be expressed in blood cells. As it is easier to obtain human PBLs in comparison with human tissues, I therefore used the 9A11 mAb to screen PBLs to characterize the cellular expression profile of hCLEC9A.

Human PBMCs were stained with 9A11 (Figure 4.9 A), but the yield of positive cells was very low, approximately ~0.4% of total PBMC were 9A11 positive. Due to the low percentage of hCLEC9A expression on PBMCs, I subsequently resorted to sorting the $9A11^{+}$ cells by flow cytometry in order to enrich for hCLEC9A on human peripheral blood. Furthermore, cDNA was made from RNA extracted from $9A11^{+}$ and $9A11^{-}$ sorted cells, and hCLEC9A primers were used for subsequent RT-PCR of isolated cells.

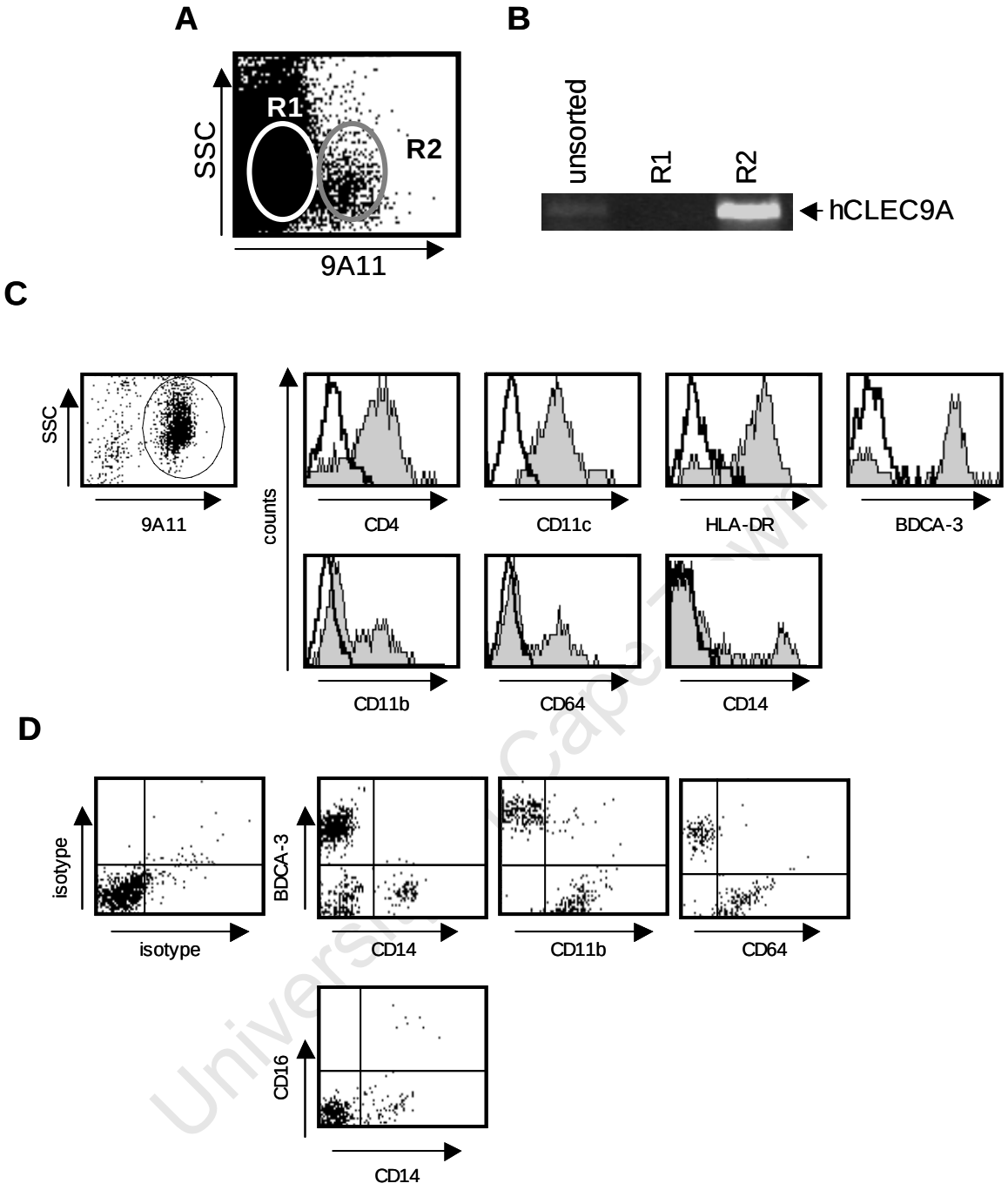


Fig. 4.9 Expression of CLEC9A on BDCA3$^+$DC and a subset of CD14$^+$CD16$^-$ monocytes. A) Dot plot showing SSC versus 9A11 staining of PBLs. R1 and R2 indicate the 9A11$^-$ and 9A11$^+$ cell populations, respectively, isolated by cell sorting and analyzed for hCLEC9A expression by RT-PCR **(B).** PBMC cells expressing this receptor were sorted, prior to analysis by multicolor flow cytometery **(C)**. Using various markers, as indicated, all CLEC9A$^+$ cells (gated) were found to be CD4$^+$HLA-DR$^+$CD11c$^+$. **D)** Further analysis of the 9A11$^+$ cells indicated that the receptor was predominantly expressed on BDCA3$^+$ dendritic cells, but also on a subset of CD64$^+$CD11b$^+$CD14$^+$CD16$^-$ monocytes and a third population of CD64$^+$CD11b$^+$CD14$^-$CD16$^-$ cells which were not identified further. The data shown are representative of data obtained from at least six different donors.

The PCR results show that hCLEC9A is present in the 9A11^{+}sorted cells and absent in the 9A11^{-} sorted cells (Figure 4.9 B). The results confirmed that we have enriched for the hCLEC9A expressing cells. The hCLEC9A 9A11^{+} sorted cells obtained from PBMCs were stained with various antibodies specific for different subsets of cells to characterize the cells that hCLEC9A is expressed on. Single staining with selected markers showed that hCLEC9A^{+} cells expressed CD4, CD11c and HLA-DR (Figure 4.9 C) The majority of the hCLEC9A^{+} sorted cells were BDCA-3^{+} [196], and a subpopulation of CD11b^{+}, CD64^{+} and CD14^{+} cells were also detected (Figure 4.9 C) Double staining with these markers revealed that hCLEC9A is expressed on three populations of cells, BDCA-3^{+} DCs, CD11b^{+}/CD64^{+}/CD14^{+}/CD16^{-} monocytes, and a CD11b^{+}/CD64^{+}/CD14^{-}/CD16^{-} population of cells which we were unable to identify further (Figure 4.9 D). The hCLEC9A^{+}sorted cells were negative for the expression of CD1a, CD1c, CD2, CD8, CD34, CD123, BDCA-1, BDCA-2, DC-SIGN as well as lineage specific markers including CD3, CD19 and CD56 (Figure 4.10). Further analysis of PBMCs indicate that hCLEC9A was expressed on all BDCA-3^{+}cells, 5% (±2%) of all CD11b^{+} cells, 2% (±1) of all CD14^{+} cells, and 6% (±3%) of all CD64^{+} cells (data not shown).

Thus the expression of hCLEC9A was apparent on two distinct subpopulations of cells. The major population of hCLEC9A positive cells including a type II myeloid DCs [197] (CD1a^{-}/CD4^{+}/CD11c^{+}/HLA-DR^{+}/BDCA-3^{+}) and the second minor population the "classical monocytes" [198] (CD11d^{+}/CD14^{+}/CD16^{-}/CD64^{+}) based on the FACS data (Figure 4.9 C and D).

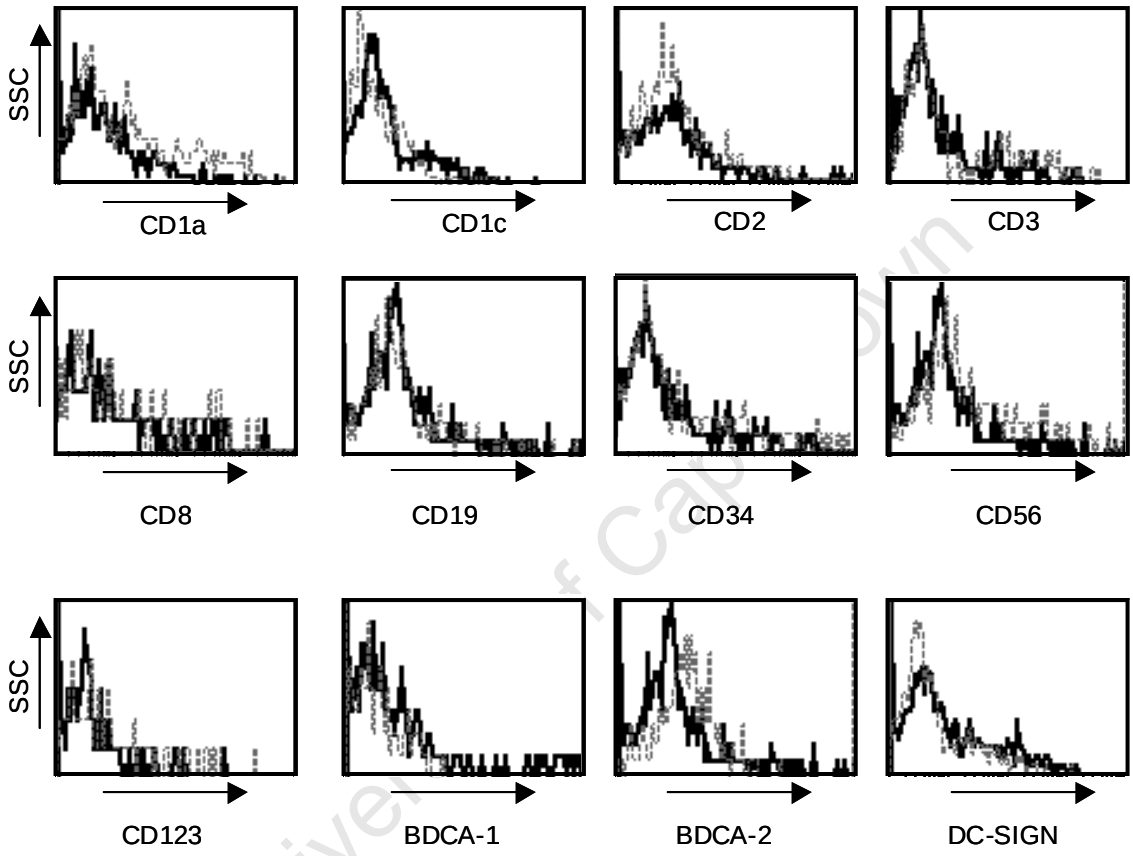


Fig. 4.10 Human CLEC9A staining on various PBMCs. Histograms show sorted 9A11 cells counterstained for various leukocytes, T-cells, B cells, NK cells blood DC subset markers (grey) or isotype-matched control (black). hCLEC9A were absent in all of these stains. The data shown are representative of data obtained from at least six different donors.

4.2.6 hCLEC9A expression on *in vitro* cultured macrophages and DCs, and tissues

Circulating monocytes are recruited to tissues where they mature into macrophages and DCs [199]. Since hCLEC9A was found on myeloid type II DCs and a small subset of

monocytes, the effects of maturation on hCLEC9A expression in peripheral blood monocytes was also investigated. The maturation process can be mimicked by *in vitro* culture of monocytes over several days (see *Matarials and Methods*). Monocytes cultured with GM-CSF and interleukin-4 (IL-4) induce a DC-like phenotype [200]. DCs become activated during infections *in vivo*, which trigger their migration to lymph nodes and enhance their maturation, an effect which can be mimicked *in vitro* by stimulation with LPS.

Cultured buffy coat monocytes were analyzed by live flow cytometry with 9A11 and other myeloid markers to confirm their phenotype (Figure 4.11). At day 1, monocytes isolated from peripheral blood were positive for CD14 (Figure 4.11 1st row). DC markers such as DC-SIGN are downregulated on these monocytes. Upon maturation towards macrophages, most cells are CD14^{+}, while DC markers such as DC-SIGN and CD86 remain low or were downregulated (Figure 4.11 2nd and 3rd row). On the contrary, cultured buffy coat monocytes stimulated with GM-CSF and IL-4 showed that CD14^{+} expression was slightly downregulated after four days, while the DC marker DC-SIGN was significantly upregulated (Figure 4.11 4th row), indicating that these cells were immature myeloid dendritic cells [201]. Following LPS-induced activation of cultured DCs, the co-stimulatory molecule CD86 was upregulated and CD14^{+} expression was lost (Figure 4.11 5th row), indicating that these cells represent mature DCs.

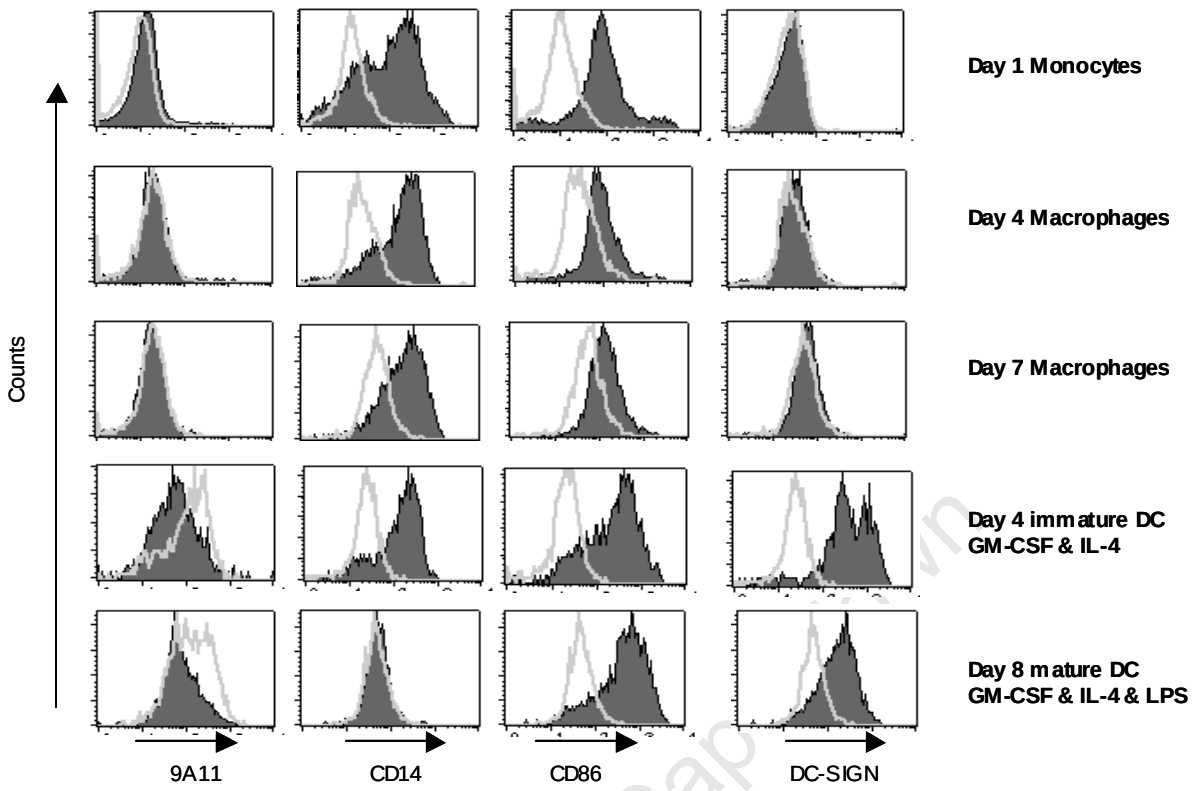


Fig. 4.11 Expression of hCLEC9A on monocyte-derived macrophages and DC. Live (surface only) staining by flow cytometry of monocytes (1st row), isolated form total peripheral blood mononuclear cells by adherence, that were cultured and differentiated *in vitro* into macrophages (analysed after 4 days (2nd row) or 7 days (3rd row) or, with GM-CSF and IL-4, into immature DCs (analysed at day 4, 4th row) or mature/activated DCs (by the addition of LPS at day 6 and analysis at day 8, 5th row). The various markers analysed are indicated at the bottom of each column and their staining is represented by the filled histograms, unfilled histograms represent staining with isotype-matched control antibodies. The classical markers of monocytes/macrophages/DCs behaved as expected and confirm the different phenotypes of the cells, while the hCLEC9A was absent on *in vitro* cultured monocytes, macrophages and DCs. Each experiment is representative of 3 donors.

However, the surface expression of hCLEC9A was absent from all *in vitro* cultured, peripheral blood-derived, macrophages and DCs (Figure 4.11). Isotype-matched negative

controls for each antibody used were also included. Thus hCLEC9A is not expressed on in vitro cultured macrophages and DCs derived from peripheral blood.

4.3 Discussion

This chapter described the characterization of the expression profile of CLEC9A. The results showed that hCLEC9A mRNA was broadly expressed as a single transcript in most organs, but predominantly in the brain, thymus and spleen. This is different to the mCLEC9A, which encodes at least five isoforms, but was also broadly expressed in most organs, including heart, spleen, skeletal muscle and day 11 embryo. Human Dectin-1 has two major isoforms and six minor isoforms, the two major isoforms are the only isoforms functional for binding β-glucans [74, 100]. However, these two major isoforms are differently expressed in various cell types, indicating that the alternative splicing of these specific isoforms can be regulated [74]. The physiological function and cellular distribution of these mCLEC9A isoforms still needs to be determined. Both human and murine CLEC9A were expressed and transported to the cell surface. hCLEC9A was expressed as a homodimer and N-glycosylated, whereas the mCLEC9A was expressed as a non-glycosylated monomer. The results confirmed the predictions made in chapter 3, which predicted that hCLEC9A possesses a single predicted N-glycosylation site (81 NFTE) in the stalk region (Figure 3.2 B). Whereas the predictions made for mCLEC9A is inconsistent with the result obtained, there were two predicted N-glycosylation sites for mCLEC9A, one in the stalk region and one in the CTLD (Figure 3.2 B).

As mentioned previously Sancho *et al.* [191] and Caminshi et al. [190], identified and characterized the mCLEC9A version containing seven exons, and subsequently reported

that it is expressed as a dimer, although they did not confirm N-glycosylation. They also identified only three isoforms of mCLEC9A. This is in contrast to our findings, but we used the six exon mCLEC9A sequence for the characterization assays, which could explain the difference in results obtained. However all of the 'Dectin-1 cluster' receptors consist of six exons. The seven exon version of mCLEC9A contains an 'extra' exon in the CTLD (see Figure 4.2 B referred to as mCLEC9Aβ) and consists of 264 amino acids. This elongated version of mCLEC9A contains the residues involved for dimerization, which is absent in the six exon version of mCLEC9A. This is a possible explanation for the difference in dimerization between six and seven exon versions of mCLEC9A.

To study the expression of CLEC9A in more detail we generated a novel mAb against hCLEC9A. The functional characterization of hCLEC9A on primary cells was limited by its expression on extremely rare populations of PBLs and the lack of expression on human tissues. Five distinct subsets of human blood DCs have been reported, including a population of $CD123^+$ plasmacytoid DC (pDC) and different subsets of putatively myeloid $CD123^-$ DC distinguishable on the basis of expression of CD16, CD1b/c, BDCA-3, and CD34 [202]. The $9A11^+$sorted cells were negative for CD123, indicating that hCLEC9A is not expressed on pDC (Figure 4.10). hCLEC9A expression was also negative for CD34, CD16 and CD1b/c (Figure 4.10). The hCLEC9A receptor was predominantly expressed by $BCDA\text{-}3^+$ DC, which represents less than 0.05% of PBMCs [197]. There is not much known about this cell type, although they are thought to be immature precursors of interstitial DCs and were recently shown to express multiple Toll-like receptors [197, 202, 203]. $BDCA3^+$ DCs share similarities with mouse $CD8\alpha^+$ DCs

[204], the mouse CD8α^{+} DCs is known to excel in MHC class I cross-presentation of cell-associated foreign and self antigens [205-208]. The expression of hCLEC9A on BDCA3^{+} cells was subsequently confirmed by Sancho *et al.* [191] and Caminschi *et al*. [190]. Thus hCLEC9A serves as a novel marker for BDCA-3^{+} cells.

Caminschi *et al*. [190] showed that hCLEC9A was expressed on a proportion of B cells, whereas no expression of hCLEC9A were detected on B cells by our group or by Sancho *et al.* [191]. Their expression results of hCLEC9A on B cells were very low and they did not elaborate on this finding.

Two major human monocyte subsets have been published, including a population of CD14^{hi}, CD11b^{hi}, CD11c^{hi} positive cells but CD2^{-} (T cell marker), CD19^{-}/CD24^{-} (B cell marker), BDCA-2^{-}/ CD123^{-} (interferon producing cell marker) and CD2^{-}/CD56^{-} (NK cell marker) negative cells which correspond to the "classical monocytes". The second population of monocytes are characterized as being CD14^{lo}CD16^{hi}, CD11b^{+} and CD11c^{+} but negative for T, B, NK and interferon-producing cell markers corresponding to the CD16^{+} defined monocytes [209]. hCLEC9A is expressed on the "classical monocytes", because the 9A11^{+} sorted cells were negative for CD16. CD14^{+}/CD16^{-} monocytes, the so-called "classical monocytes", which normally constitute around 14% of the PBMCs are thought to migrate to sites of inflammation where they can differentiate into DCs [198, 209]. The expression of hCLEC9A on a minor subset of these cells is suggestive of further differentiation of this "classical monocytes" population to possibly mature into

BDCA-3^{+} blood DCs, and this receptor could potentially be used as a marker to characterize this subset in future analyses.

I did not characterize the cellular distribution of mCLEC9A. However, Caminschi *et al.* [190] and Sancho *et al.* [191] both show that mCLEC9A was specific for mouse DCs and was restricted to the CD8α^{+} conventional DC (cDC) and at a significantly lower level on pDCs by antibody detection. Future RT-PCR studies should elucidate the relative expression profiles of the various splice variants in specific cell populations. Thus CLEC9A serves as a novel marker to distinguish subtypes of both human and mouse DCs.

Chapter Five

Functional studies

5.1 Introduction

Cell surface receptors on DCs are important for the recognition, communication, migration and activation of DCs. Surface molecules that vary between DC subtypes are of great interest, since they may support the functional specialisation of these specific subtypes. Surface receptors that differ between DC subtypes may identify and also serve as a selective delivery for antigens or therapeutic agents to DCs, in order to manipulate immune responses. Several antibodies against DC surface molecules, such as DEC-205, which have been conjugated with an adjuvant, have been used for immunotherapy of cancer or for boosting cellular immunity to infection [210-213]. Successful targeting depends on suitable DC-expressed cell surface receptors that mediate endocytosis of bound antibodies. This endocytic process will allow delivery of antibodies to endosomal compartments where their associated antigens can be processed for MHC presentation. Potential receptors include the MMR and Fcγ receptors that are expressed on DCs and other cell types. Some receptors which are more restricted to DCs include DEC-205, DC-SIGN, Langerin, asialoglycoprotein receptor and BDCA-2 [45, 214, 215]. Most of these receptors belong to the C-type lectin family and may have a role in antigen-uptake, which could be exploited for targeting [33, 46].

Many C-type lectins act as phagocytic receptors in myeloid cells. Myeloid CTLRs often contain distinct internalisation motifs in their cytoplasmic tails including tri-acidic amino acids, di-leucine motifs and tyrosine-based motifs, which can direct both endocytosis and/or phagocytosis of its ligand and subsequent sorting of the receptor and its ligand [216]. Dectin-1 is an example of such a receptor that mediates phagocytosis of yeast and

yeast-derived particles such as zymosan, through the tri-acidic motif and the tyrosine residue in its cytoplasmic region, which is also required for endocytosis [51, 106, 120]. The intracellular pathway taken by a specific CTLR can be dependent on the receptor sequence motif and on the nature of the ligand [46]. For example, in DCs and fibroblasts expressing mDectin-1, the phagocytosis of zymosan is partly dependent on the tyrosine kinase Syk [106, 120]. However, in macrophages, mDectin-1 mediated phagocytosis is independent of Syk, yet the production of reactive oxygen species (ROS) requires this kinase [51, 106].

The aim of this chapter is to characterize the physiological function of hCLEC9A. The cytoplasmic tail of hCLEC9A contains a putative internalisation motif and an ITAM-like motif (Figure 3.1 B) [51], which resembles the classical ITAMs that are usually associated with phagocytosis and activatory receptors [124]. These features make it possible to speculate that hCLEC9A can act as a potential endocytic receptor and/or the presence of an ITAM-like motif suggests that hCLEC9A is capable of mediating phagocytosis. Therefore we were interested whether hCLEC9A can mediate these processes. Signaling from the cytoplasmic tail of hCLEC9A was also investigated. The kinases associating with the ITAM of hCLEC9A were identified, and we determined that this is an activatory receptor with the ability to produce proinflammatory cytokines after receptor stimulation.

5.2 Results

5.2.1 Endocytic ability of hCLEC9A

Given the limitation in obtaining human primary CLEC9A^{+} cells for functional analysis, we resorted to determining the function of this receptor in transduced cell lines. To examine if hCLEC9A has the ability to mediate endocytosis, we utilized NIH3T3 fibroblasts stably expressing hCLEC9A. Antibody mediated cross-linking of CLEC9A on the surface of transduced fibroblasts which were incubated at 4°C or 37°C, respectively, for different lengths of time before staining with a fluorescent-labeled secondary antibody. Flow cytometry was used to determine the surface expression of hCLEC9A. The surface expression of hCLEC9A was already reduced by more than 50% after 30-minute incubation, with minimal decrease in surface expression at 4°C (Figure 5.1 A), indicating that the receptor was mediating internalization of the bound antibodies. Dectin-1, which is also known to mediate endocytosis [51] was included as a positive control (Figure 5.1 B).

In order to establish whether the observations made in the fibroblasts could be applied to a phagocytic cell line, the same experiment as described above was conducted in RAW264.7 macrophages. Similar data were obtained in transduced RAW264.7 macrophages stably expressing hCLEC9A (Figure 5.2 B). The surface expression of hCLEC9A was significantly reduced following cross-linking at 37°C compared to cells maintained at 4°C. In order to elucidate the intracellular fate of hCLEC9A following antibody-induced internalization, RAW264.7 transduced cells expressing hCLEC9A

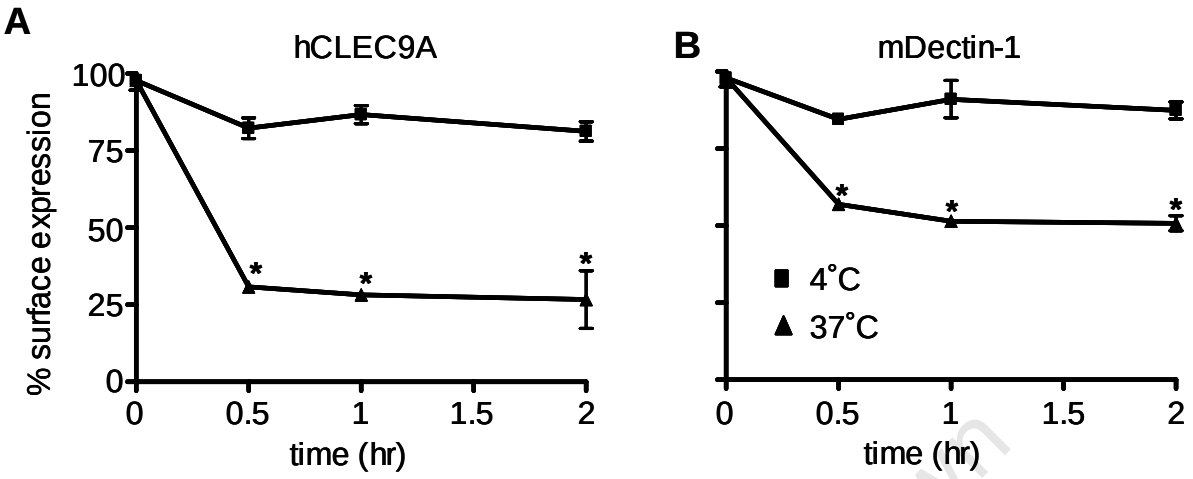
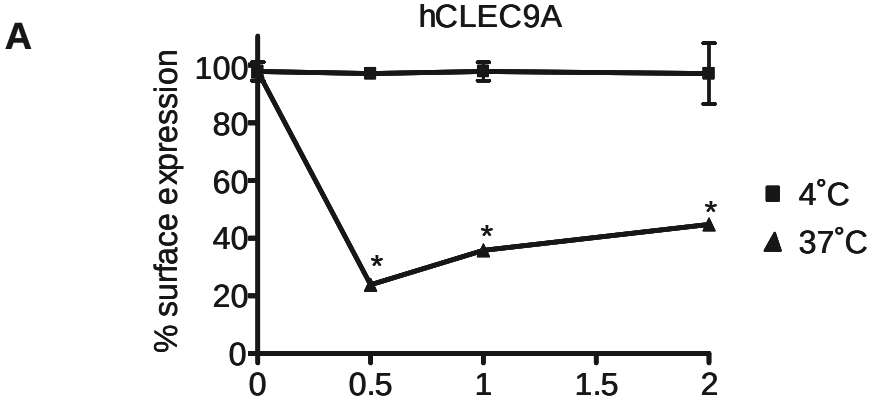


Fig. 5.1 hCLEC9A is an endocytic receptor. A) Internalization of CLEC9A expressed in NIH3T3 fibroblasts, following antibody crosslinking, was determined by flow cytometry. The analysis, performed by comparing cells incubated at 37°C versus 4°C (to prevent internalization), demonstrates that hCLEC9A is rapidly removed from the cell surface following crosslinking, indicative of receptor endocytosis. **B)** Dectin-1, previously shown to be endocytic[90] was included as a control. The data shown are mean ± SD of triplicates, and representative of three independent experiments. *, $p < 0.05$ *versus* control (Student's *t* test).



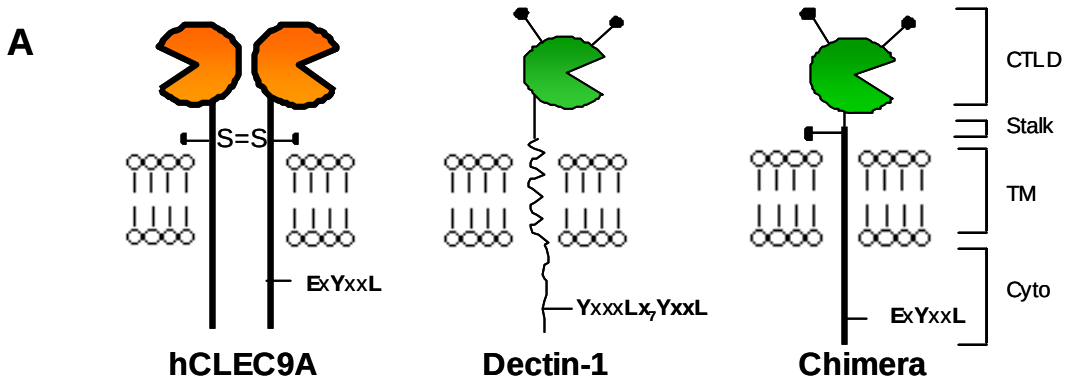
were stained with anti-CLEC9A and co-stained with the late endosomal/lysosomal marker, LAMP-1, and analyzed by fluorescent microscopy. Fluorescent microscopy of cells stained with anti-CLEC9A antibody demonstrated the formation of intracellular

CLEC9A^{+} endocytic vesicles. Some intracellular hCLEC9A co-localized with LAMP-1 (Figure 5.2 B) indicating that hCLEC9A could mediate delivery of bound antibody to the endocytic/lysosomal pathway.

5.2.2 Phagocytic function of hCLEC9A

Since the ligand for hCLEC9A is still unknown, we generated a chimeric receptor described in *Materials and Methods*, which could bind the Dectin-1 ligand, zymosan, and which was used to study the phagocytic ability of hCLEC9A. This chimeric receptor consisted of the cytoplasmic tail, transmembrane region and part of the stalk of hCLEC9A fused with the CTLD of mDectin-1 (Figure 5.3 A). RAW264.7 macrophages stably expressing this monomeric chimera would allow us to trigger hCLEC9A signaling using zymosan [29]. This chimeric construct was expressed at the cell surface as well as the full-length hCLEC9A and mDectin-1 receptors, which were included as controls, as detected by flow cytometry using RAW264.7 macrophages (Figure 5.3 B). A vector-only cell line was included as a negative control.

After confirming the cell surface expression of these transduced macrophages, we wanted to examine the ability of the chimera to functionally bind zymosan, using mDectin-1 as a positive control [29]. Cells transfected with hCLEC9A and vector only were unable to bind zymosan, whereas the chimera transfectants were able to bind zymosan to a level

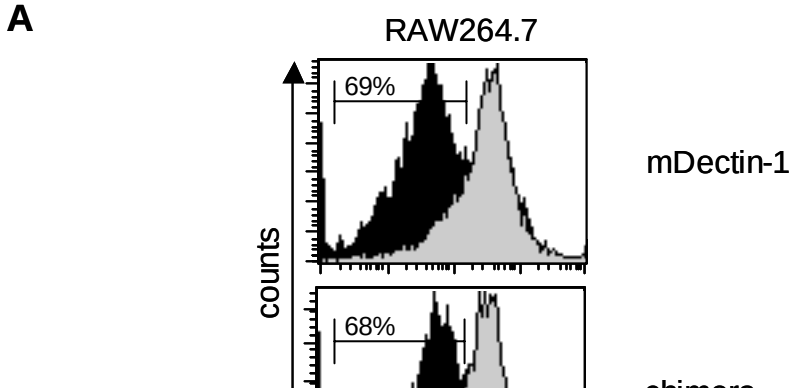


comparable with that of cells expressing mDectin-1 (Figure 5.3 C). The Dectin-1 dependency of this binding was confirmed by specific blocking with the soluble β-

glucan, glucan phosphate. Full-length hCLEC9A receptor does not bind zymosan (Figure 5.3 C).

Dectin-1 requires its ITAM-like motif to mediate particle uptake [51]. Therefore cells transfected with the chimera were used to examine their ability to internalize zymosan, via the cytoplasmic tail of hCLEC9A. RAW264.7 transfectants expressing the chimeric receptor and Dectin-1 bound zymosan equally well (Figure 5.3 C). Anti-zymosan flow cytometric assay show that both chimera and mDectin-1 could internalize over 65% of the zymosan it bound (Figure 5.4 A). Some cells were also treated with cytochalasin D to inhibit actin polymerisation, and prevent particle uptake, and were used as controls in these experiment. Furthermore, by fluorescence microscopy, FITC-labeled zymosan could be seen inside macrophages expressing the chimeric receptor and Dectin-1 (Figure 5.4 C). Thus, the chimeric and Dectin-1 transfectants could mediate particle uptake in an actin-dependant manner in macrophages.

Characterization of receptor mediated phagocytosis is generally performed in non-myeloid cell lines to avoid confounding factors, such as antibody mediated recognition through macrophage Fc receptors as well as to demonstrate that the receptor can confer phagocytic activity in normally non-phagocytic cells. The phagocytic capacity of receptors, such as the Fc receptors, mannose receptor, Dectin-1 and CLECSF8 for example, have all been characterized in this way [47, 51, 217, 218].



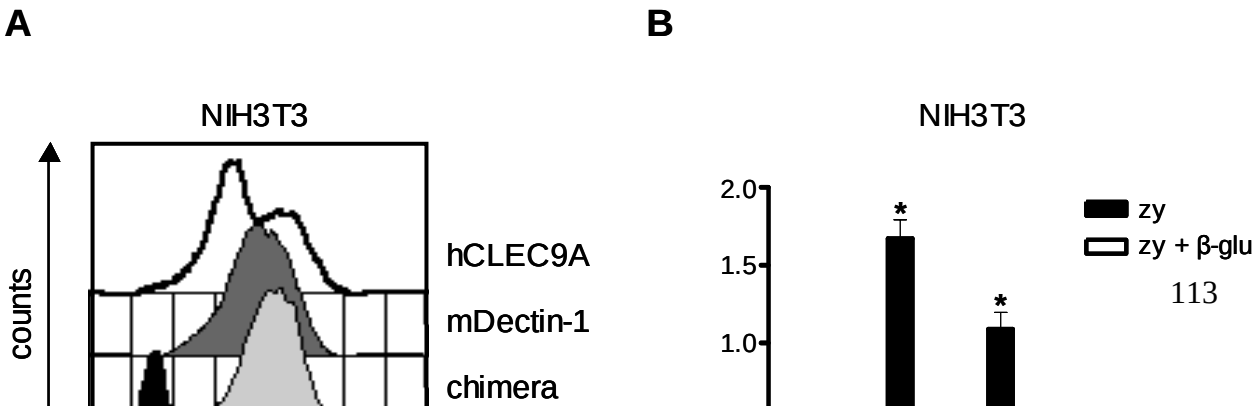
Therefore we expressed the chimeric receptor in NIH3T3 cells, which is a non-phagocytic cell line, and examined the ability of these cells to internalize zymosan. The

surface expression of each construct was confirmed by flow cytometry (Figure 5.5 A). As before Dectin-1 was included as a positive control.

The transduced-fibroblasts expressing the chimera and Dectin-1, but not vector only or full-length hCLEC9A, conferred the ability of these cells to bind zymosan in a β-glucan dependent fashion (Figure 5.5 B). Over 50% of Dectin-1 transfected fibroblasts were able to internalize the bound FITC-labeled zymosan, whereas the chimeric receptor was unable to internalize their zymosan significantly above control levels (Figure 5.5 C). As demonstrated by fluorescence microscopy, Dectin-1 expressing fibroblasts internalized FITC-labeled zymosan, while the chimeric receptor was unable to mediated particle uptake in these cells, as the bound FITC-labeled zymosan could not be internalized by the chimeric transfectants (Figure 5.5 D). Thus, hCLEC9A is not a true phagocytic receptor, as it is unable to mediate particle uptake in a non-phagocytic cell line.

5.2.3 hCLEC9A is an activation receptor

In addition to phagocytosis, the tyrosine-based motif of Dectin-1 can induce a variety of cellular responses, including the production of proinflammatory cytokines in response to zymosan [219]. Therefore I wanted to explore the possibility that signaling via hCLEC9A could induce TNF-α production in RAW264.7 macrophages transduced with the chimeric receptor.



5.2.3.1 Cytokine production by hCLEC9A

To explore the possibility that hCLEC9A can induce TNF-α production in the RAW264.7 macrophages we utilized the chimeric receptor. The full-length hCLEC9A, vector-only and Dectin-1 cell lines, which were included as controls and the surface expression of each construct was confirmed by flow cytometry (as shown previously in Figure 5.3 B). As described before, expression of Dectin-1 and the chimeric receptor resulted in increased levels of zymosan binding in these macrophages (Figure 5.3 C). The transduced-macrophages were stimulated with zymosan and after three hours the supernatants were collected for measuring TNF-α. Dectin-1 [119] and the chimeric receptor resulted in high levels of TNF-α production in response to zymosan (Figure 5.6 A). This was confirmed to be a β-glucan specific response since the response could be blocked by glucan phosphate (a soluble β-glucan polymer) [220]. In a previous study it was shown that Dectin-1 lacking a cytoplasmic tail can mediate zymosan binding but not the induction of TNF [119]. The results therefore indicate that the cellular response induced by the chimeric receptor is due to the presence of the hCLEC9A cytoplasmic tail. Thus, like Dectin-1, hCLEC9A signaling can induce a proinflammatory response.

5.2.3.2 The kinases associated with the ITAM-like motif of hCLEC9A

As cytokine induction mediated by Dectin-1 involves signaling via Syk [122, 219], we examined the possibility that this kinase was involved in the intracellular signaling mediated by hCLEC9A. In order to determine if Syk was recruited by the ITAM of hCLEC9A, tyrosine-phosphorylated and unphosphorylated peptides corresponding to the cytoplasmic tails of hCLEC9A were generated. Dectin-1 peptides were included as

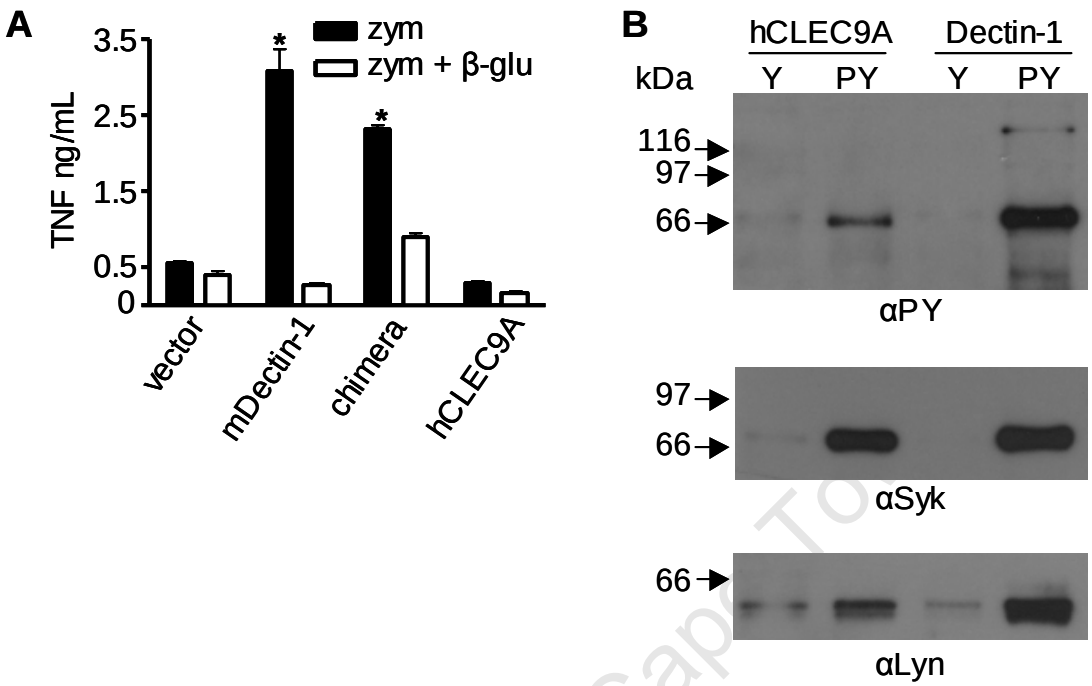


Fig. 5.6 hCLEC9A can induce pro-inflammatory cytokine production and can recruit and signal via Syk kinase. A) Quantitation of zymosan (zym) induced TNF production by transduced RAW264.7 macrophages, in the presence or absence of soluble β-glucan (β-glu), as indicated, demonstrating the ability of the chimeric receptor to induce proinflammatory cytokine production. Shown are the mean ± SD of one representative experiment of three. *, $p < 0.05$ *versus* control (Student's *t* test). **B)** Western blotting of immunoprecipitates using phosphorylated (pY) and unphosphorylated peptides (Y) corresponding to the cytoplasmic tail of CLEC9A or Dectin-1 from RAW264.7 cell lysates. Blots were probed with anti-phosphotyrosine (αPY), anti-Syk and anti-Lyn, as indicated.

controls [120]. These peptides were then used to immunoprecipitate associated signaling molecules from RAW264.7 cell lysates, which were subsequently analyzed by Western blotting with specific antibodies (Figure 5.6 B). Probing with an anti-phosphotyrosine antibody indicated a predominant band of approximately 70kDa in immunoprecipitates with phosphorylated peptides of both hCLEC9A and Dectin-1. These bands were absent in the immunoprecipitates of unphosphorylated hCLEC9A and Dectin-1 peptides (Figure 5.6 B). Probing with an anti-Syk antibody revealed that the one of the bands seen in the anti-phosphotyrosine blot was Syk with a molecular mass of 72kDa (Figure 5.6 B). Further blotting with various antibodies showed that hCLEC9A associated with the src kinase, Lyn, which is thought to be involved in tyrosine phosphorylation of ITAM motifs upon ligand binding [221]. These results indicate that hCLEC9A associates with Syk and Lyn kinases.

5.2.4 hCLEC9A is Syk dependent for activation

The results show that hCLEC9A is an activating receptor with the ability to produce pro-inflammatory cytokines, while immunoprecipitates show that hCLEC9A associate with Syk. Therefore, the next step was to confirm that hCLEC9A could induce intracellular signaling via Syk kinase. To examine this possibility Syk-sufficient (Syk^+) and Syk-deficient (Syk^-) B-cell lines were transfected with the chimeric receptor. This approach was similar to that which had been used previously to demonstrate the Syk-dependent activity of Dectin-1 [120]. The chimeric receptor was similarly expressed at the cell surface of both cell types as determined by anti-2A11 flow cytometery, which detect the CTLD of Dectin-1 (Figure 5.7 A). The addition of zymosan to the Syk^+ cells expressing

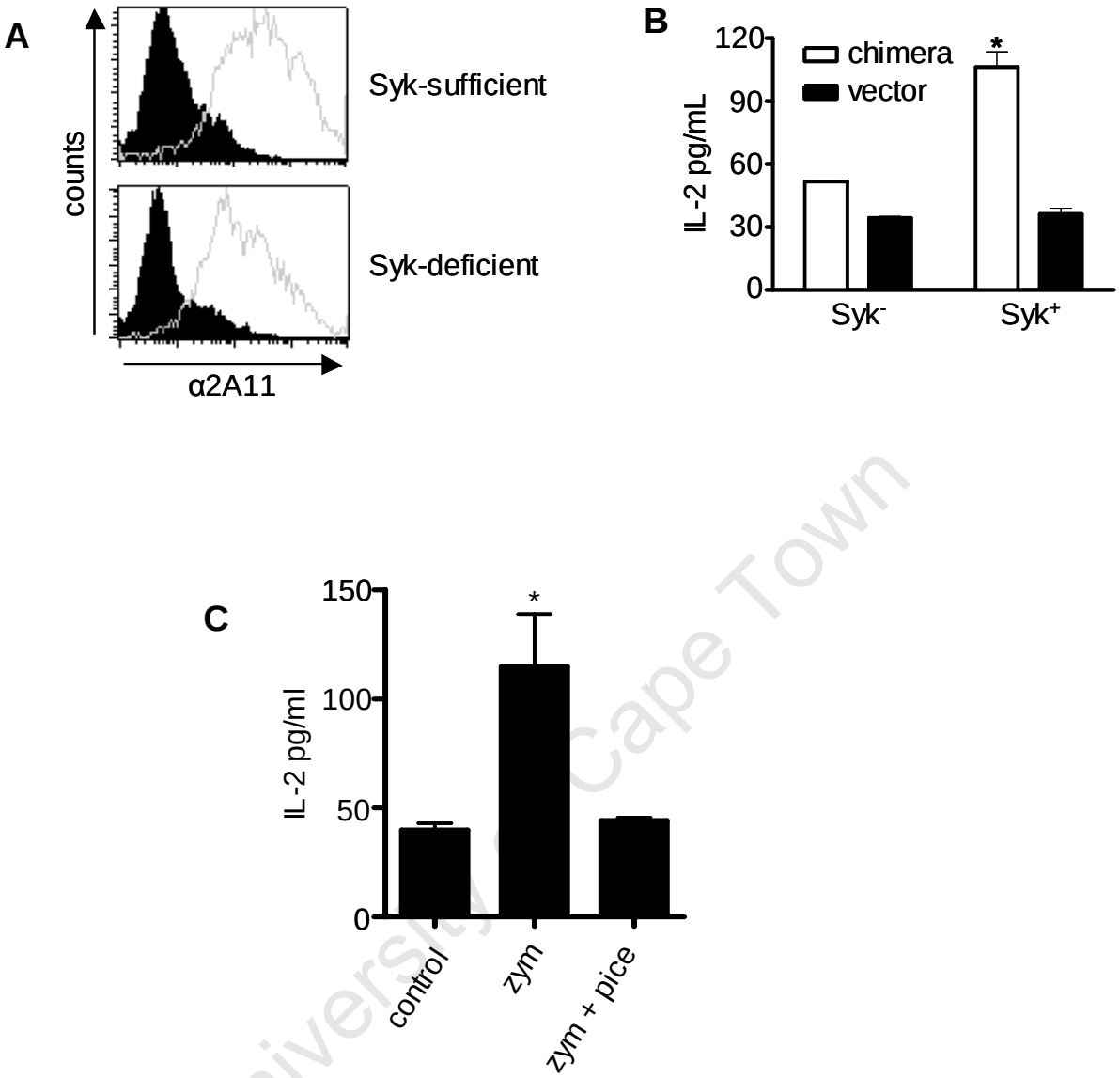


Fig. 5.7 hCLEC9A is Syk dependent for activation. A) Expression of the chimeric receptor (gray line), detected with αDectin-1, on the surface of Syk sufficient and Syk deficient cells, as indicated. The filled histograms indicate vector only controls. **B)** IL-2 production following zymosan stimulation of Syk-deficient (Syk^-) and Syk-sufficient (Syk^+) cells, transduced with the chimeric receptor or vector only control, as indicated, showing that cytokine production induced by the chimeric receptor in response to zymosan requires Syk. The data shown are the mean ± SD and are representative of three independent experiments. *, $p < 0.05$ *versus* control (Student's *t* test). **C)** IL-2 production following zymosan (zym) stimulation in the Syk^+ B-cells, can be inhibited with piceatennol (pice). *, $p < 0.05$ *versus* control (Student's *t* test).

the chimeric receptor induced significant amounts of IL-2 production (Figure 5.7 B). In contrast, this response was absent in vector control transduced cells and in the transduced Syk^- cells. Furthermore, the IL-2 response could also be significantly inhibited in the Syk^+ cells by including the Syk-inhibitor piceatannol (Figure 5.7 C). Thus, hCLEC9A can mediate intracellular signaling via Syk kinase.

5.3 Discussion

The chapter has described the characterization of possible functions of hCLEC9A by identifying elements of the cytoplamic tail. NIH3T3 fibroblasts and RAW264.7 macrophages were transduced with the chimeric receptor (consisting of the cytoplasmic tail of hCLEC9A and the CTLD of Dectin-1) to probe the intracellular function of hCLEC9A, as a ligand for hCLEC9A was not available. A novel anti-hCLEC9A mAb was also used, to determine the endocytic ability of this receptor in using retrovirally transduced cell lines containing the full-length hCLEC9A receptor.

Sequence analysis predicted that the cytoplasmic tail of hCLEC9A possesses a putative internalization motif. The functional characterization of this receptor revealed that hCLEC9A mediates endocytosis in both fibroblast and macrophage transfected cell lines. The results were confirmed by Sancho *et al*. [191]: where they show that CLEC9A mediates endocytosis of bound antibody in BDCA-3^+ DCs, as well as endocytosis in CD8α^+ DCs.

The restricted expression and endocytic ability of CLEC9A suggests that this receptor may provide a suitable target for antibody-mediated antigen delivery to mouse CD8α$^+$ DCs or human BDCA-3$^+$ DCs, as has been demonstrated for a variety of other C-type lectins, including Dectin-1 [222]. Subsequently, it was shown that mCLEC9A-targeted antigens are efficiently cross-presented to CD8$^+$ T cells [191], indicating that, like the MR and DEC205 [210, 223], CLEC9A can deliver its cargo to selected endosomal compartments that intersect with the MHC processing and presentation pathway. This is consistent with the observation that anti-CLEC9A antibody can be endocytosed by both human and mouse DCs and concentrate in the intracellular vesicles.

Mouse CD8α$^+$ DCs have the ability to capture material from dying cells [224, 225] and process these cell-associated foreign and self-antigens for MHC class I cross-presentation for the activation of CD8 T cells [205-208]. The activation of CD8α$^+$ DCs leads to the production of IL-12 [226, 227] and are able to induce inflammatory Th1 responses [228, 229]. Alternatively, CD8α$^+$ DCs are also capable of effectively activating CD4 T cells and this feature most probably represents the role of these cells in enhancing antibody responses. The restricted expression of mCLEC9A on CD8α$^+$ DCs [190, 191] and its endocytic ability suggest that CLEC9A could be used for antigen targeting to DCs. Recently it was shown that antigens covalently coupled to anti-mouse CLEC9A are selectively cross-presented by CD8α$^+$ DCs *in vivo* and when given along with an adjuvant, it induced potent cytotoxic T-lymphocyte (CTL) responses [191]. These CTL responses could facilitate the prevention of tumor development, as well as mediating

tumor eradication [191]. The results indicate that CLEC9A could be exploited for CTL cross-priming and tumor therapy.

hCLEC9A could also induce particle uptake in macrophages, but not when expressed in fibroblasts. By definition a phagocytic receptor is able to confer phagocytic activity to normally non-phagocytic cell lines [47, 51, 217, 218]. Therefore, it was concluded that CLEC9A is not directly able to mediate particle uptake. The phagocytosis of zymosan particles can involve several macrophage receptors including Dectin-1 [102], FcγRs [218] and the human MR [47, 217]. In addition, residual low levels of endogenous Dectin-1 is expressed on RAW264.7 macrophages, which could be contributing to the phagocytosis process [51]. Dectin-1 also mediates particle uptake through a novel undefined mechanism, which requires additional tyrosine-proximal residues [106], these residues are absent in hCLEC9A.

Despite the lack of an hCLEC9A ligand, it was still possible to further investigate the function of the signaling motif in the cytoplasmic tail of hCLEC9A using the chimeric receptor. Analysis of the predicted hCLEC9A polypeptide sequence (see section 3.1) predicted that the cytoplasmic tail contains an ITAM-like motif. The results demonstrate for the first time that hCLEC9A is an activation receptor, capable of triggering intracellular signaling via Syk kinase. This interaction is mediated by the cytoplasmic tail of hCLEC9A that contains a single tyrosine-based (YxxL) sequence, which can be considered to be an ‘ITAM-like’ motif [51]. These sequence motifs were first identified in other closely related receptors that are part of the “Dectin-1 cluster”, Dectin-1 itself

[120], and CLEC-2 [125]. The mechanism of Syk signaling via these motifs is unknown though it does require both SH2 domains of this kinase [124]. ITAM-like mediated signaling through Syk has largely been studied in myeloid cells with Dectin-1. Signaling via Syk occurs through a novel pathway involving CARD9 [127], and can induce a variety of cytokines (such as TNF, IL-6, IL-10, IL-23 and IL-2), the respiratory burst and the production of arachidonic acid [103, 219]. As hCLEC9A can signal via this pathway, there is a possibility that this receptor may be able to mediate some of these responses in DCs.

Subsequently, it was also shown that none of the anti-CLEC9A antibodies are able to promote DC activation *in vitro* or *in vivo* as measured by the upregulation of co-stimulatory molecules or induction of cytokines [190, 191]. Furthermore, treatment with anti-CLEC9A mAbs does not alter the response of DCs to poly I:C or CD40 ligation, suggesting that anti-CLEC9A mAbs do not provoke signaling through the receptor, at least in a soluble form [191]. Antibodies to Dectin-1, similarly do not act as agonists for its receptor, the reason for this may be that the antibodies do not elicit the degree of cross-linking required to achieve productive signaling [103].

All the results put together suggest that CLEC9A mediate the uptake of antibodies and promote antigen cross-presentation in a restricted subset of DCs, and the activatory potential of this receptor could potentially lead to various downstream signaling events that could initiate inflammatory responses that play a key role in tumor rejection or for boosting cellular immunity to infections.

Chapter Six

Ligand screen for mCLEC9A

6.1 Introduction

Since the release of the human and mouse genome sequences, many novel ligand-orphan cell surface receptors have been identified. As indicated by sequence analyses, CLEC9A is most homologous to the "Dectin-1 cluster" receptors. Dectin-1, LOX-1 and CLEC-2 have been reported to bind a diverse, unrelated range of endogenous and exogenous ligands, whilst CLEC-1, CLEC12B and MICL have no published ligands. It is therefore not clear what a ligand for CLEC9A might be based on its shared homology to related molecules.

Given the limitation in obtaining primary hCLEC9A$^+$ cells for analysis and the difficulty obtaining ethical approval for human tissues for ligand screening, I resorted to focusing on the murine orthologue to continue elucidating the physiological role of the receptor in the immune system. This chapter describes my investigation into the identifying possible endogenous and exogenous ligand(s) of mCLEC9A.

6.2 Results

6.2.1 Ligand screen of mCLEC9A using reporter cells

The first approach involved expressing mCLEC9A in a reporter cell line. This approach employs BWZ.36 cells containing a β-gal reporter construct that is activated via NFAT, and is an approach that has previously been utilized to identify ligands for several mouse

CTLRs [73, 175]. BWZ.36 cells were retrovirally transduced to express a chimeric mCLEC9A/CD3ξ receptor and in these reporter cells, ligand binding to the mCLEC9A CTLD induces signaling through the CD3ξ cytoplasmic tail as well as the induction of IL-2 and β-galactosidase (Figure 6.1 A). As a mAb against mCLEC9A was not available, the surface expression of the mCLEC9A transfectants cells was detected by using a purified polyclonal antibody raised in rats against mCLEC9A. Serum collected prior to the immunization of rats was used as a negative control, referred to as pre-bleed. The cell surface expression of this construct as well as a control chimera of mDectin-1/CD3ξ was confirmed by flow cytometry (Figure 6.1 B).

Single cell suspensions isolated from various mouse tissues were co-cultured with the reporter cells overnight at 37°C. After the overnight incubation period the supernatants were collected and tested for the production of IL-2 by ELISA (Figure 6.1 C). By comparison with the Dectin-1 reporter cells, which were used as a control, significant induction of IL-2 was observed in the lung, heart, brain, kidney and bone marrow and to a lesser degree in spleen and liver. Similar results were obtained by the β-galactosidase staining (Figure 6.2), which indicating that the induction of IL-2 was not induced by the tissues themselves but from a specific endogenous ligand express on these cells (shown in Figure 6.1 C). These data therefore suggest that mCLEC9A recognizes an endogenous ligand(s), broadly expressed in most of mouse tissues.

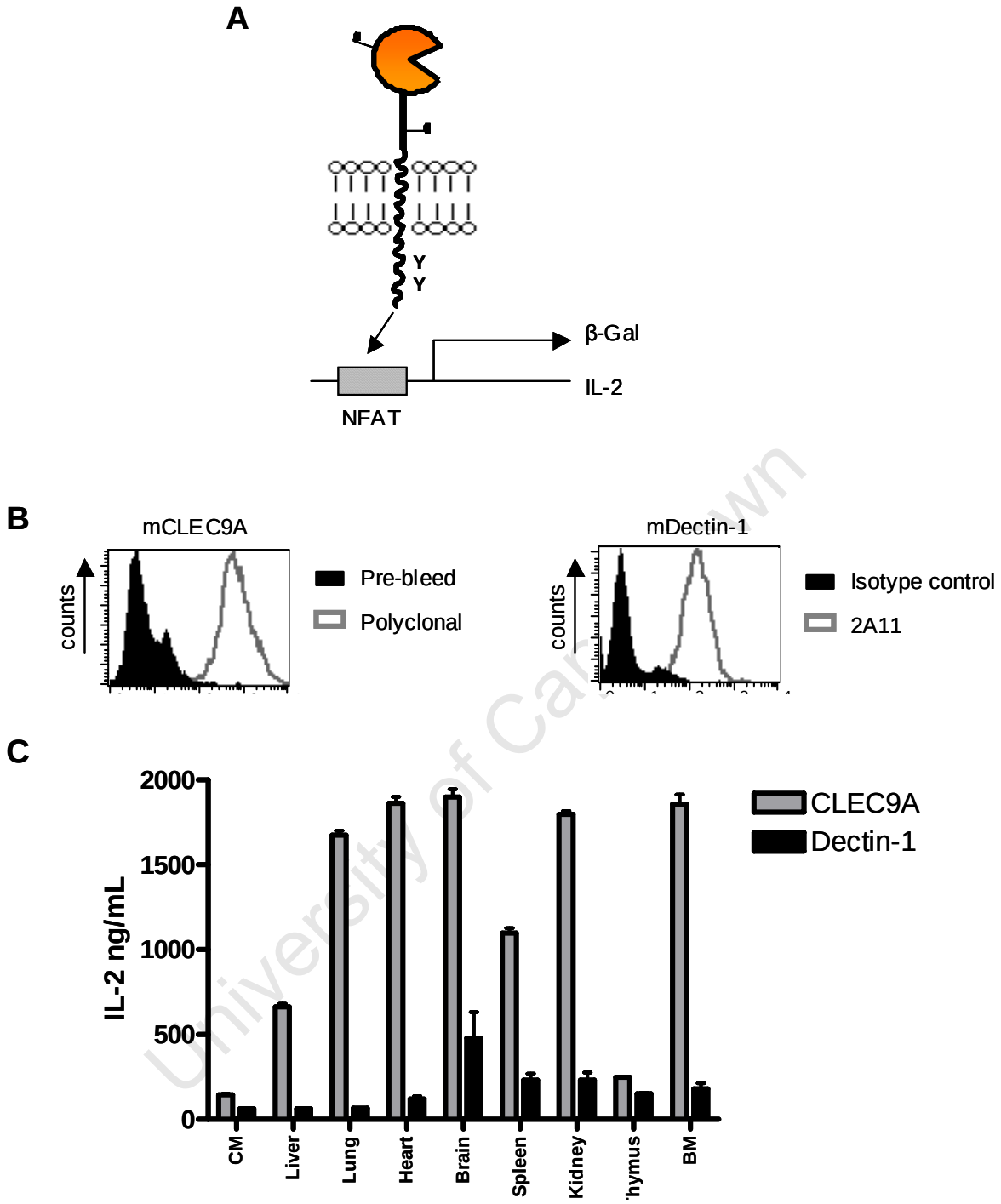


Fig. 6.1 mCLEC9A recognises an endogenous ligand. **A)** Cartoon representation of the CLEC9A/CD3ζ chimeric receptor and signal transduction pathway in BWZ.36 reporter cells. **B)** Flow cytometry of BWZ.36 cells stably expressing mCLEC9A or mDectin-1/ CD3ζ chimeric receptors stained with anti-mCLEC9A polyclonal antibody and anti-mDectin mAb (2A11). The filled histograms indicate isotype controls. **C)** IL-2 production from BWZ.36 reporter cells after co-culture with single cell suspensions isolated from various murine organs, as indicated. Dectin-1 (black) was included as a control.

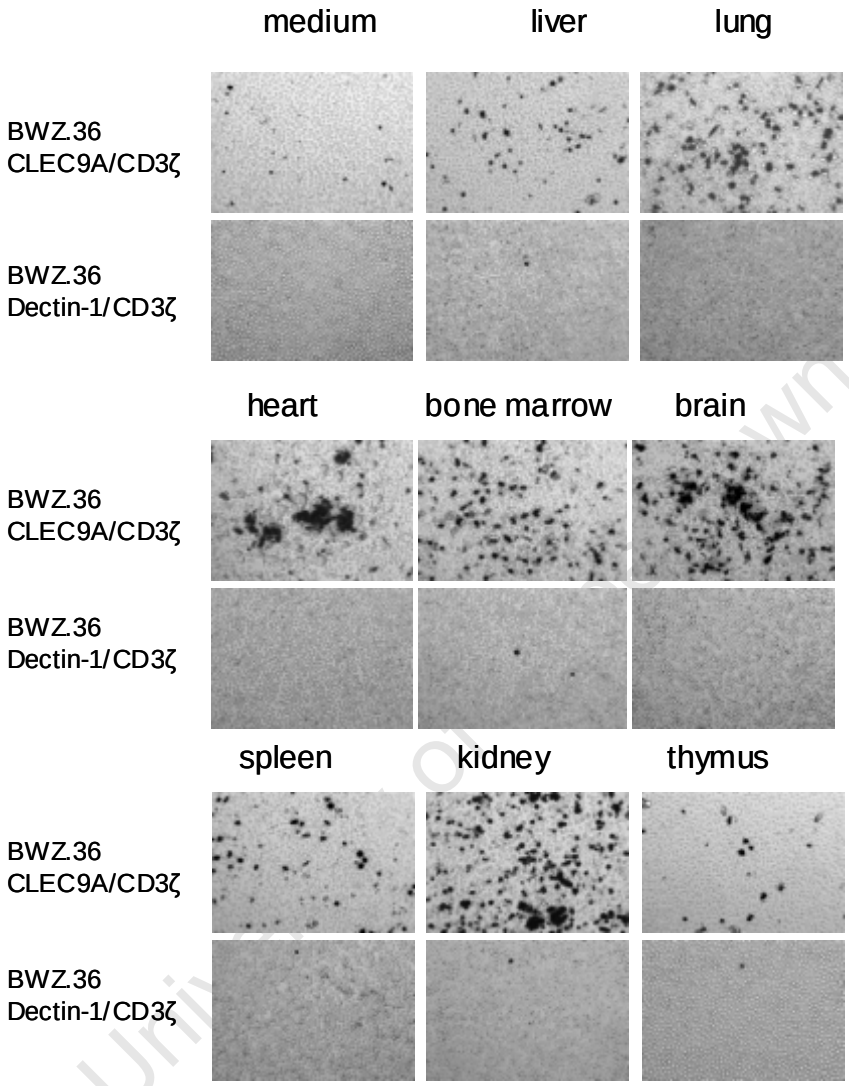


Fig. 6.2 X-gal staining of reporter cells. BWZ.36 reporter cells expressing mCLEC9A (upper panel) and Dectin-1 (lower panel) were stimulated with single cell suspensions isolated from various mouse organs, as indicated.

We next used cellular suspensions at various concentrations to test for the production of the IL-2 over time at 3, 6 and 20 hours of incubation at 37°C (Figure 6.3). The optimal time point for IL-2 production was after 20 hours of incubation at 37°C using $1x10^6$ cells from each mouse tissue. The results obtained in the optimization of IL-2 induction (Figure 6.3) were similar to the results shown in Figure 6.1 C and 6.2. The lung, bone marrow, kidney, heart, spleen and brain once again tested positive for the induction of IL-2, while the liver and thymus did not induce significant levels of IL-2.

6.2.2 Screening tissues with an Fc-mCLEC9A soluble protein

An alternative method was used to probe cells isolated from numerous mouse tissues by flow cytometry, using a soluble Fc-mCLEC9A fusion, similar to the human Fc-CLEC9A protein described in chapter 4. Using this approach, we could detect limited binding of Fc-CLEC9A to cells isolated from brain, kidney and lung (Figure 6.4). This interaction appeared to be specific, as similar binding was not obtained with Fc-CLEC12B, which was used as a control. These results confirmed the result obtained from the IL-2 and β-galactosidase assay, however, there was no binding of Fc-mCLEC9A to liver, thymus, bone marrow, heart and spleen detected by flow cytometry.

6.2.3 Immunohistochemistry of tissues stained with Fc-mCLEC9A

Various mouse organs were collected and sectioned as described in *Material as Methods*. These tissues sections were stained with Fc-mCLEC9A as well as Fc-mCLEC12, which was included as a control. The binding of Fc-proteins was detected using anti-human Cy3 (red) and the nuclei were stained with DAPI (blue) (Figure 6.5).

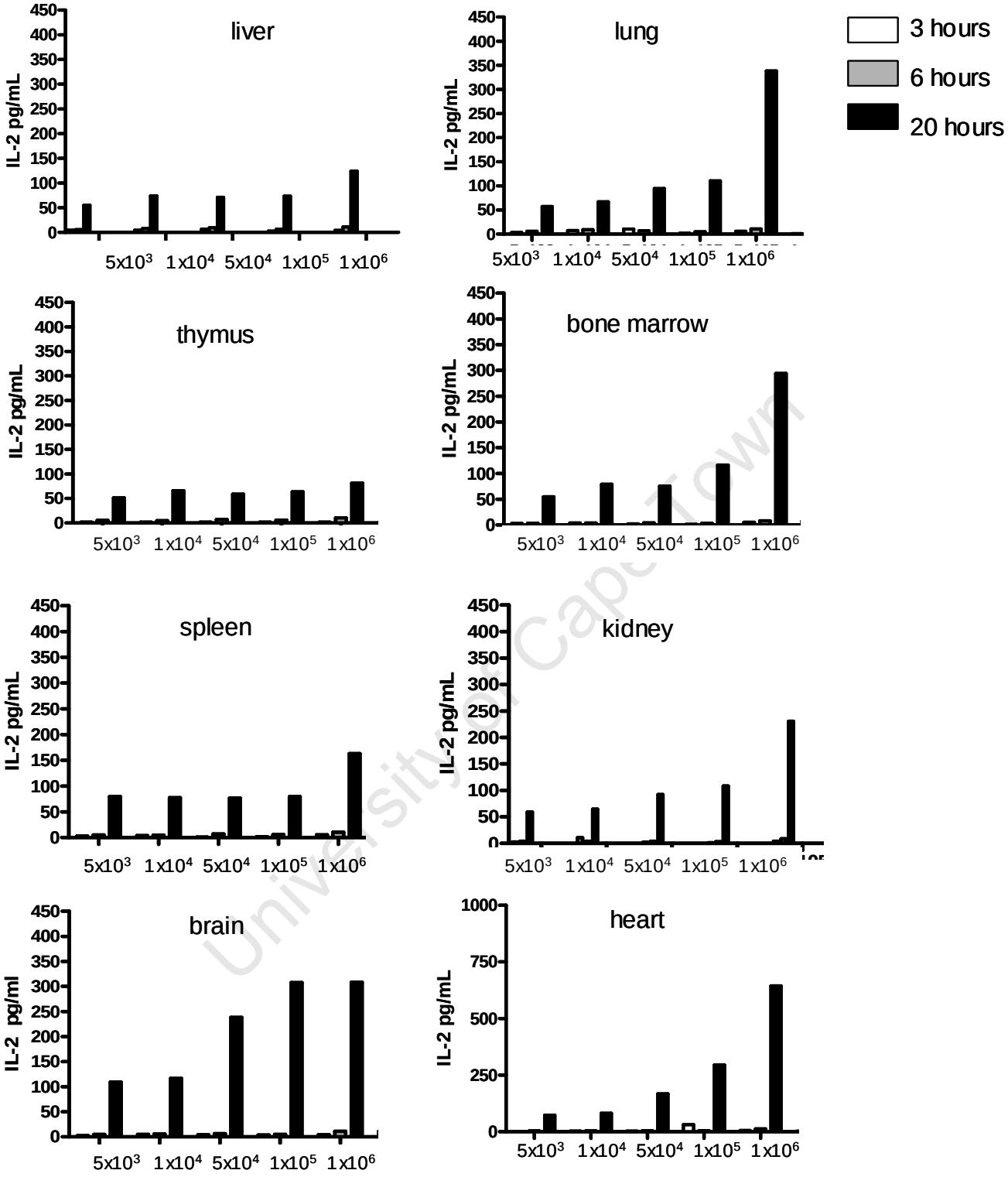


Fig. 6.3 Cellular titration of various mouse tissues and different time points for IL-2 production. IL-2 production from BWZ.36 reporter cells after co-culture with single cells suspensions, at different concentrations as indicated, of various mouse tissues over time (3hrs, 6hrs and 20hrs).

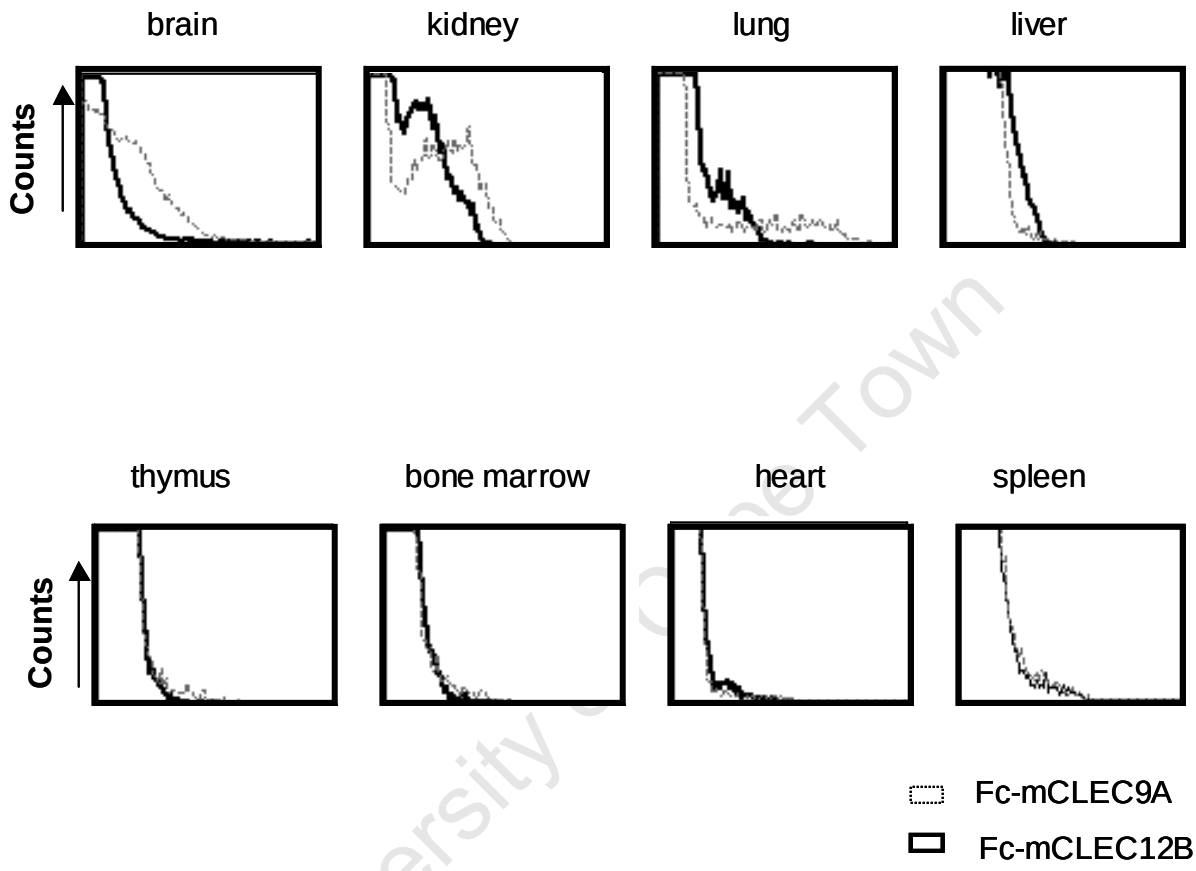


Fig. 6.4 mCLEC9A recognises an endogenous ligand. Flow cytometric analysis of live single cell suspensions isolated from various murine tissues, as indicated, and stained with Fc-mCLEC9A (dotted grey line) and Fc-CLEC12B (solid black line) was included as a control. The data are representative of three independent experiments.

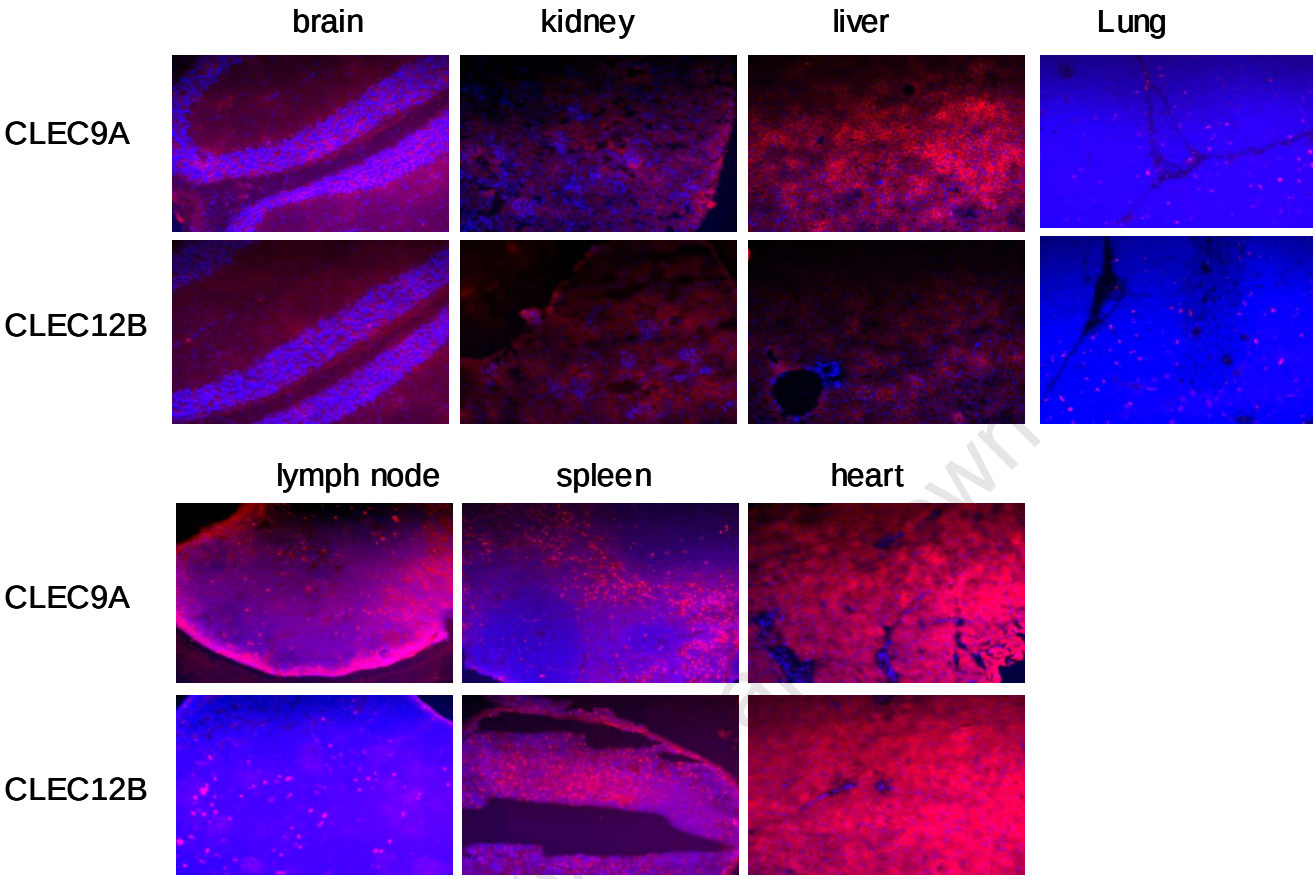


Figure 6.5 Immunohistochemistry of various mouse organs stained with Fc-CLEC9A. Various tissues were stained with either Fc-mCLEC9A or mCLEC12B. Staining was detected with anti-human Cy3 (red) and nuclei were stained with DAPI (blue).

Unfortunately the staining of these tissue sections with the Fc-proteins was nonspecific. I attempted to optimize the fixation method of the tissues, but was unable to get rid of the nonspecific binding.

6.2.4 Screening various heat-killed pathogens as possible exogenous ligands

The mCLEC9A/CD3ξ reporter cells were also used to screen for any exogenous ligand(s). The reporter cells were co-cultured with various heat-killed pathogens, including *E. coli*, *Pneumocystis*, *M. Tuberculosis*, *H.influenzae*, *S. aureus*, zymosan and *N. meningitidis*. After the 20-hour incubation period at 37°C the supernatants were collected and tested for the production of IL-2 by ELISA. There was no induction of IL-2 detected for any of the microbial ligands tested as shown in Figure 6.6, suggesting that the receptor does not function as a PRR. Dectin-1 was included as a control and showed increased IL-2 production upon zymosan binding, as expected [29]. Unstimulated cells were also included as a negative control. The β-galactosidase staining was used to verify the IL-2 results and confirmed that mCLEC9A did not recognize any of the pathogens tested (data not shown).

6.2.5 Screening soluble Fc-proteins as possible ligands

Recently AICL (CLEC12B), an activating CTLR expressed on myeloid cells [230], was identified as a ligand for NKp80, which is a NK cell-specific stimulatory receptor [231, 232]. NKp80 and AICL are both NKC-encoded receptors, the NKp80-AICL interactions are involved in activating the cross-talk between NK cells and myeloid cells and could

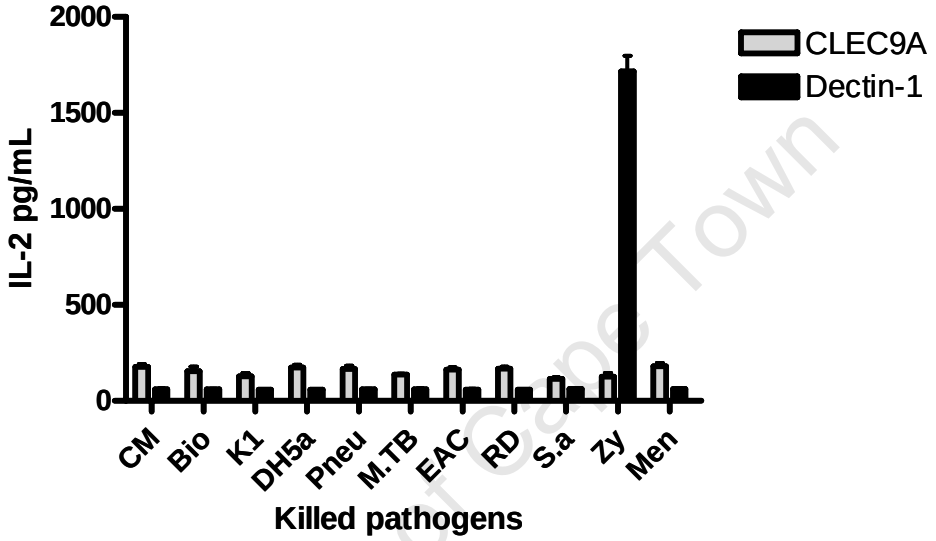


Fig. 6.6 mCLEC9A does not recognise an exogenous ligand. IL-2 production from BWZ.36 reporter cells after co-culture with various heat killed pathogens (CM: culture medium; Bio: *E.coli* bio particles; K1: *E.coli*; DH5a: *E.coli*; Pneu: Pneumocystis; M.TB: Killed M. Tuberculosis; EAC: H.influenzae; RD: H.influenzae; S.a: *S.aureus*; Zy: zymosan; Men: N.meningitidis; as indicated. Dectin-1 (black) was included as a control and showed increased IL-2 production upon zymosan binding.

potentially influence the initiation and maintenance of immune responses [230]. Previously, Nkrp1d, which is a NK cell receptor was shown to recognize the specific C-type lectin-related ligand Clrb, which is expressed on DCs and macrophages [73]. These receptor-ligand pairs are co-localized in the centromeric end of the NKC, and their gene order is in opposite transcriptional orientation [73]. Therefore, it might be possible that CLEC9A might be genetically linked to a C-type lectin-related ligand in the 'Dectin-1 cluster'. Since the main focus of our laboratory is to functionally characterize CTLRs that are part of the 'Dectin-1 cluster', we had the advantage of having all the protein constructs and soluble Fc-proteins for Dectin-1, CLEC-1, CLEC-2, MICL and CLEC12B available to use for screening as possible ligands for CLEC9A. To explore this, the reporter cells (BWZ.36 mCLEC9A/ mCD3ζ) were incubated with the soluble Fc-proteins at 4°C and binding was detected by anti-Fc flow cytometry (Figure 6.7). All of these Fc-proteins tested negative. Therefore the results indicate that mCLEC9A does not recognize any of these specific CTLRs as an endogenous ligand.

6.3 Discussion

This chapter describes the identification of possible endogenous and exogenous ligand(s) for mCLEC9A. Various approached were attempted in order to find ligands for mCLEC9A, this involved the screening of a variety of mouse tissues and heat-killed pathogens. The specific ligand for mCLEC9A was not identified, however, some results did indicate that mCLEC9A recognized an endogenous ligand(s) in brain, kidney and lung. This was confirmed by using two approaches, the reporter cell system and anti-Fc-mCLEC9A flow cytometric assays. These screening methods tested will be exploited for

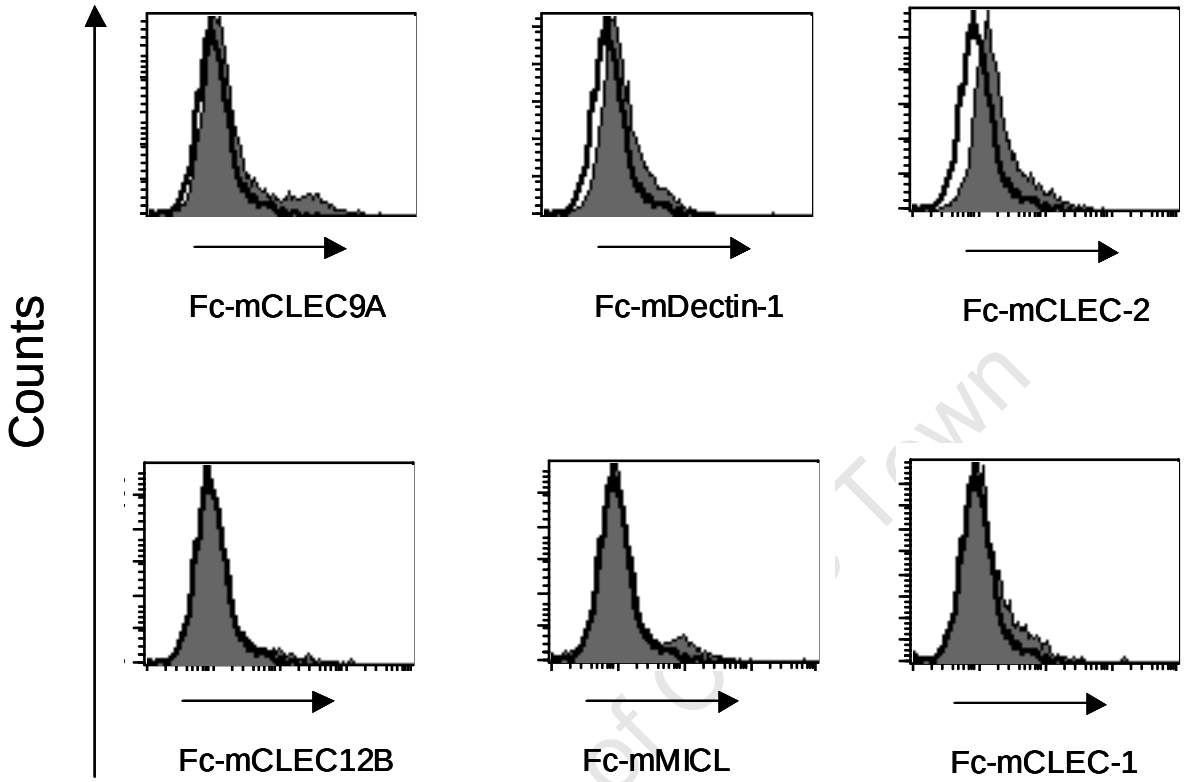


Fig. 6.7 BWZ.36-mCD3ζ/mCLEC9A transduced cells stained with various soluble Fc-proteins. The transduced BWZ.36 cell line was stained with mCLEC9A, Dectin-1, CLEC-2, CLEC12B, MICL and CLEC-1 to look for possible ligands. All the Fc-proteins tested negative. The black line is the Fc-protein and the solid grey line is the isotype control.

future identification of specific endogenous mCLEC9A ligand(s). Using methods to increase activity of binding such as those used to demonstrate the presence of a cell surface ligand for EMR3 on macrophages and neutrophils [233], could be used for future studies to enhance ligand detection. In our hands we could not detect any microbial ligands for mCLEC9A, suggesting that this receptor is unlikely to function as a PRR.

It is known that the ligands of the 'Dectin-1 cluster' are structurally unrelated. Dectin-1 binds β-glucans as well as an unknown endogenous T cell antigen [29, 39]; LOX-1 recognizes aged cells, bacteria, activated platelets and Ox-LDL [52, 87], whilst CLEC-2 binds to the endogenous ligand podoplanin [77] as well as the snake venom toxin rhodocytin which elicits a powerful platelet activation response [101]. There is also some evidence for an endogenous ligand for mMICL [175]. Therefore, we could not predict what the ligand for CLEC9A might be by comparing the receptor homology with receptors of known ligands. It is even possible that CLEC9A could bind other C-type lectins that are not part of the 'Dectin-1 cluster'

The reporter cells were very useful for identifying which organs might be used for future ligand searches, although this approach does not give any detail as to the specific endogenous ligand recognized by mCLEC9A. A future approach could utilize flow cytometric cell sorting to isolate the cells that bind Fc-mCLEC9A. Once the positive populations of Fc-binding cells are obtained, these cells could either be stained with various specific cell markers to identify the cellular population that express the endogenous ligand or used in immunoprecipitations. Once the cellular population is

identified these cells could be sorted and cell lysates generated from them and analyzed by immunoprecipitations assays using anti-FcCLEC9A as a probe, and various proteomic approaches, including mass spectrometry, could be used to define the specific endogenous ligand for mCLEC9A.

Some activatory CTLRs bind exogenous ligands, however I could not detect any binding of mCLEC9A to the microbes used for in these experiments. It may be that fixation of the pathogens tested, damaged the ligand for mCLEC9A, so live microbes might be used for future experiments. Since CLEC9A also shares high homology to LOX-1, a potential ligand(s) that could be tested using the same approach are apoptotic/aged cells, activated platelets and heat shock proteins (Hsps), which have all been reported as ligands of LOX-1. Another possibility is that CLEC9A functions as part of a complex, which could be a problem if using the above mentioned screening approach, because the reporter cells expressing mCLEC9A is an artificial system that might lack the molecules needed for binding its natural ligand(s). In such an event, primary cells expressing mCLEC9A, such as $CD8\alpha^+$ DCs, would need to be assayed for the identification of a CLEC9A-dependent interaction with ligand-expressing cells.

DCs utilize several pathways in antigen capture such as macropinocytosis; receptor-mediated endocytosis via CTLRs (including MMR and DEC-205) or Fcγ receptors (CD64 and CD32) for the uptake of opsonized particles; and phagocytosis of particles such as apoptotic and necrotic cell fragments (involving CD36 and integrins), viruses and bacteria (reference) as well as intracellular parasites [228]. Furthermore, DCs can also

internalize the peptide loaded Hsps g96 and Hsp70 through unknown mechanisms [234]. The endocytic ability and restricted expression of CLEC9A on CD8α^{+}DCs suggest that this receptor could mediate antigen capture of dying cells [207, 224, 225] for antigen processing and presentation [224]. This observation could be exploited in future studies to identify cell-associated foreign and self-antigens as possible endogenous ligand(s) for CLEC9A.

Chapter Seven

General discussion

7.1 Review of aims of the project

The aim of this thesis was to characterize a novel group V C-type lectin-like receptor, CLEC9A, which is part of the 'Dectin-1 cluster' [171]. In this chapter I will discuss my results obtained from my characterization of the receptor's polypeptide sequence, its mRNA and protein expression, its signaling abilities, and its ligand(s) to ultimately elucidate the role that this receptor plays in the immune system.

7.2 Summary of key findings

7.2.1 CLEC9A is a novel human CTLR with an ITAM-like motif

The sequence analysis of CLEC9A indicates that it is part of the 'Dectin-1 cluster' of receptors that belong to the group V CTLRs. CLEC9A lacks the calcium coordinating amino acid residues found in the classical C-type lectins [8]. The stalk region of hCLEC9A contains two cysteine residues that are potentially involved in receptor dimerization, and a single predicted N-linked glycosylation site. The cytoplasmic tail of CLEC9A contains a single tyrosine residue within a sequence showing similarity to the ITAM-like sequence of Dectin-1 [51], indicating that CLEC9A may act as an activatory receptor. The hCLEC9A gene consists of six exons and is located on the plus strand of chromosome 12 within the NKC.

Sequence analysis shows that the protein encoded by murine *clec9A* is 53% identical to human *clec9A,* retains most of the structural features described above, including the intracellular signaling motif. However, the murine receptor appears to lack N-glycosylation sites as well as one of the cysteine residues in the stalk region. Similar to the human gene, mCLEC9A is located on the plus strand of chromosome 6 and also includes a variety of alternatively spliced transcripts. The protein sequence of CLEC9A is most homologous to Dectin-1 and MICL. Subsequently two independent publications appeared, which also identified CLEC9A as a novel CTLRs. Sancho *et al.* [191] and Caminschi *et al*. [190] published similar findings, however they characterized a seven exon transcript of mCLEC9A in both their studies. This transcript contains an 'extra' exon in the CTLD of mCLEC9A and encoding a protein of 264 amino acids. All the 'Dectin-1 cluster' receptors consist of six exons, therefore I decided to characterize the six exon transcript encoding a protein of 238 amino acids.

7.2.2 hCLEC9A is predominantly expressed as a single transcript on blood DC and a subset of monocytes

At the mRNA level, hCLEC9A is expressed in most tissues but predominantly in brain, thymus and spleen with no apparent splice variants. The high level of hCLEC9A expression in the thymus and spleen, may suggest a role in immunity. Multiple transcripts of mCLEC9A were detected in spleen, lung and day 11 embryo, however in most other tissues mCLEC9A appears to be expressed as a single transcript. We used a mAb generated against hCLEC9A to further characterize the expression of hCLEC9A in peripheral blood. hCLEC9A expression was detected on an extremely rare population of

peripheral blood leukocytes. The receptor is predominantly expressed by BDCA-3^+ DCs, which represent less than 0.05% of PBMCs [197]. The surface expression of hCLEC9A on BDCA-3^+ DCs was confirmed by Sancho *et al.* [191] and Caminschi *et al.* [190]. hCLEC9A is also expressed on small subsets of $CD14^+CD16^-$ monocytes and on $CD64^+CD11b^+CD14^-$ cells, which we were unable to identify further. I did not characterize the expression of mCLEC9A on primary cells, but Sancho *et al.* [191] and Caminschi *et al.* [190] showed that mCLEC9A is expressed on $CD8\alpha^+$ DCs and at much lower levels on pDCs.

7.2.3 hCLEC9A protein is expressed at the cell surface as a dimer and is N-glycosylated

hCLEC9A is expressed at the cell surface, similar to the other receptors in the "Dectin-1 cluster", suggesting that hCLEC9A does not require an adaptor molecule for expression. The hCLEC9A receptor is highly N-glycosylated similar to MICL, but bears no O-glycosylation. Like most group V CTLRs, hCLEC9A appears to be expressed as a dimer. In contrast, mCLEC9A was detected as a monomer that bears no N-/O- glycosylation, but is similarly expressed at the cell surface of transfected cells. Sancho et al. [191] reported that their seven exon molecule exists as a dimer and predicted it to be glycosylated.

7.2.4 hCLEC9A can associate and signal via Syk kinase and is able to transmit activatory signals via its ITAM

hCLEC9A functions as an activation receptor capable of triggering intracellular signaling via Syk kinase. This interaction was mediated by the cytoplasmic tail, which possesses a single tyrosine based (YxxL) sequence, which can be considered to be an "ITAM-like

motif" [124]. These conserved sequences were identified in two other receptors of the "Dectin-1 cluster", Dectin-1 itself [51], and more recently CLEC-2 [101]. The mechanism of Syk signaling via these receptors is still unknown, both SH2 domains of this kinase are required, and may occur through the bridging of two receptor chains.

7.2.5 hCLEC9A is an endocytic receptor

Cross-linking hCLEC9A on the surface of transduced cells, using anti-CLEC9A mAb, led to the rapid reduction of the receptor at the cell surface, indicating that the receptor was mediating internalization of the bound antibodies. The results show that hCLEC9A function as an endocytic receptor. The endocytic ability of human and murine CLEC9A was confirmed by Sancho *et al.* [191], primary cells and transduced cell lines were used in their experiments. This indicate that CLEC9A may be useful for targeting antigens to mouse $CD8\alpha^+$ DCs or their human equivalent BDCA-3^+ DCs

7.2.6 hCLEC9A is not a true phagocytic receptor

Since the ligand for hCLEC9A is still unknown, I utilized a chimeric receptor, which could bind zymosan to study the phagocytic ability of this receptor. Although we could demonstrate that hCLEC9A could induce particle uptake in macrophages, the receptor was unable to confer phagocytic activity in normally non-phagocytic cell lines, therefore it was concluded that CLEC9A is not directly able to mediate particle uptake. However, the assay used was based on using unphysiological cell types in an artificial system and have various limitations. Once the ligand for hCLEC9A is known physiological cells should be used to confirm the ability of CLEC9A to direct phagocytosis. CLEC9A is

expressed on DCs and may therefore require the assistance of DC-specific signaling molecules to direct phagocytosis.

7.3 Future directions

7.3.1 True physiological function

DCs consist of multiple subsets with specialized functions [235], contributing to the maintenance of self tolerance and the induction of adaptive immunity [215]. There is a definite need to identify specific surface molecules on DCs, so to manipulate distinct DCs subsets and to regulate the immune responses. This thesis describes the characterization of a novel CTLR, CLEC9A, which is restricted to human BDCA-3^+ DCs with the ability to mediate endocytosis. Subsequently, it was shown that mCLEC9A is also selectively expressed on mouse $CD8\alpha^+$ cDCs (proposed to be the equivalent of human BDCA-3^+ DCs) and pDCs [190, 191].

The conventional DCs can be distinguished as “inflammatory” DCs, which develop from monocytes upon inflammation [236]. These cDCs have been divided into those expressing $CD8\alpha^+$ ($CD8\alpha^+$ cDCs) and those that do not ($CD8\alpha^-$ cDCs). They also differ in functions, where the $CD8\alpha^+$ DCs are specialized for cross-presentation of exogenous antigens on MHC class I [205, 237, 238] as well as being major producers of IL-2 upon activation [226, 227]. Despite all the functional differences known for mouse cDCs subsets, there has been little information on the translation of this knowledge to the human DC system. However, BDCA-3^+ DCs have been previously described as a

subtype of myeloid DCs [196, 202, 239] and share similarities with mouse CD8α$^+$ DCs, including high levels of TLR3 mRNA, absence of TLR7 transcripts and expression of nectin-like protein 2 (Necl-2) [204]. BDCA-3$^+$ DCs may be the potential human equivalent of mouse CD8α$^+$ DCs. Therefore, the highly restricted expression of CLEC9A on these DCs subsets will be useful for future studies to determine whether the human CLEC9A$^+$ BDCA-3$^+$DCs have the same functions as the mouse CLEC9A$^+$ CD8α$^+$ DCs.

TLRs mainly induce DC maturation, whereas the CTLRs might act as constituents of the powerful antigen capture and uptake mechanism of DCs [33]. Several C-type lectins have the ability to endocytose a broad array of foreign material that gets targeted into lysosomal and MHC class II$^+$ late endosomes to mediate presentation to T cells, including MR, DEC-205, DC-SIGN, Dectin-1, CLEC-1 and BDCA-2 [41-43]. Specific mAbs can be used to target antigens to particular DCs surface molecules that modulate immune responses [192, 210, 214, 222]. CLEC9A is largely restricted to DCs and was used as a target for the delivery of antigens to DCs [190]. mCLEC9A expressed on DCs was targeted with a single injection of anti-mCLEC9A mAb that gave an marked increased mouse response to rat Ig, as well as the enhancement of responses to ovalbumin when conjugated to anti-mCLEC9A mAb [190]. This is a promising feature of CLEC9A if the ultimate aim is to improve antibody responses to vaccines.

Furthermore, the requirements for enhancing Ab responses by targeting mCLEC9A require that no additional DCs activation agents or adjuvants were employed. Therefore, Caminshi *et al.* [190] prepared an anti-mCLEC9A mAb under ‘endotoxin-free’

conditions to confirm that the enhanced Ab response was not due to traces of endotoxin or other microbial products, the experiments were repeated using MyD88$^{-/-}$TRIF$^{-/-}$ mice, which are unable to respond to TLR ligands [240, 241]. This indicates that the response was independent of 'danger' signals mediated by TLR ligands [190].

An equally important feature of CLEC9A are its endocytic properties suggest that it might be a useful receptor for targeting antigens specifically to mouse CD8α^{+} or human BDCA-3^{+} DCs. Recently published results show that *in vivo* delivery of modified ovalbumin peptide covalently coupled to anti-mCLEC9A mAb resulted in the selective delivery of antigen for MHC class I cross-presentation by CD8α^{+} DCs [190, 191]. Indicating that immune responses elicited by anti-mCLEC9A targeting *in vivo* primarily reflect the antigen presenting activity of CD8α^{+} DCs.

DCs have the ability to regulate adaptive immunity, which suggests that these cells could be used for clinical research through targeting antigens to DCs *in vivo* [214, 215]. The success for DC targeting depends on identifying a suitable DC-expressed cell surface receptor that mediates endocytosis. Based on these specifications Sancho *et al.* [191] assessed the potential of mCLEC9A targeting to induce CTL responses and tumor immunotheraphy. For this experiment they tested conjugates of antigen and anti-CLEC9A mAb under the cover of anti-CD40, which provides extra signals for immunogenicity [192, 210]. The results obtained were comparable to those obtained with DEC-205 where small amounts of targeted antigen similarly induce potent CTL responses, only when a DC activation stimulus were co-administered [210]. They also

applied this protocol to a tumor model, choosing B16 melanoma. Their results show that targeting antigens to mCLEC9A can eradicate tumors. These results suggest that targeting antigens to mCLEC9A could be exploited as an immunotherapeutic strategy for cancer.

These results conclude that CLEC9A is a novel activating CTLR expressed on BDCA-3^{+} DCs and is conserved between species. Together with the findings of Sancho *et al.* [191] and Caminshi *et al.* [190] our results could help to translate the information on mouse DC biology to human clinical studies. CLEC9A has the potential to be used as a target for enhancing the effectiveness of vaccines as well as improving both antibody and T cell responses to antigens. However, future studies will be needed to determine whether CLEC9A targeting induces cross-tolerance and whether CLEC9A may therefore be a promising target for promoting immunological unresponsiveness.

7.3.2 Possible ligand(s) for CLEC9A

To elucidate the true function of CLEC9A the identification of a ligand is crucial. Since I found a possible lead for an endogenous ligand by using the reporter cell method, future studies could continue using this technique as well as utilizing anti-Fc based flow cytometric assays.

Based on the restricted DC expression and endocytic function of CLEC9A, I predict that heat shock proteins (Hsps) are strong candidates to be the potential endogenous ligand(s) for this receptor. These proteins play important roles in inflammatory and immune

regulation [242, 243]. The presence of mammalian Hsps in the extracellular environment is an indication of intracellular trauma (e.g. cell death), while the detection of pathogen-derived Hsps indicates the onset of local infection [234]. Cells have the ability to sense these Hsps as molecular chaperone-based 'alarm' signals, therefore the existence of Hsp receptors has been postulated. Studies have shown that Hsps are complex ligands, once these molecules are released from the cell they often carry intracellular derived polypeptides, indicating that their receptors may recognize Hsp itself, its cargo or the Hsp-peptide complex (Hsp.PC) [244, 245]. The antigenic peptides carried by Hsp70 and gp96 (which are different classes of Hsps) stimulate a specific T cell response to the cells of the peptide origin, through antigen cross-presentation [246, 247]. Thus the specific peptide cargo of Hsps is important for different immune responses.

Hsp70 have various functions in the immune system for instance, it can bind peptide antigens, transport them to APCs and can direct antigens towards MHC class I molecules [244, 248]. Additionally Hsp70 is a potent inflammatory stimulator that triggers the innate immune system and break tolerance to tumor antigens [242]. The properties of many Hsps are transmitted through cell surface molecules and the study of Hsp receptors is still incomplete, although some important findings have been made by screening candidate hsp70 receptors. A previous study showed that Hsps70 binds readily to SCER-1 and FEEL-1 as well as LOX-1, which are all Scavenger receptors (SR). Each of these receptors can mediate Hsp70 internalization [249, 250]. LOX-1 plays an important role in immune effects of Hsp70 and can mediate cross presentation [159].

Several group V CTLRs sharing structural homology with LOX-1, including Dectin-1, NKG2D and CD94 [14] were tested for binding to Hsp70 [234], suggesting that the CTLD of these receptors consists of a conserved ligand interaction domain responsible for the recognition of Hsp70. Dectin-1 showed no significant Hsp70 binding, however NKG2D showed a significant affinity for Hsp70 [234]. These findings suggest that a number of CTLRs can serve as receptors for Hsp70, although the CTLD was not the sole determinant required for Hsp70 interaction [234].

In summary Hsps can transport antigens across the plasma membrane of APCs as cargo and deliver the antigens to the MHC class I receptors [251], leading to the activation of APC and stimulation of cytotoxic T lymphocytes [247]. Since CLEC9A is expressed on blood DC, is able to mediate endocytosis and since it can be exploited for CTL cross-priming and tumor therapy, Hsps are potential candidates to be screened as potential ligands for CLEC9A.

In conclusion, CLEC9A is a promising receptor that could be used as a novel DCs marker, could help translate the information on mouse DCs biology to human clinical application, and improve antibody responses to vaccines.

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